

Is it taking a liberty?

THE number of lectures and conferences devoted to it is growing all the time. Barely a day passes when there isn't some discussion of it on radio or television or in the newspapers. The subject is the "nuclear issue", and the immediate reason is the decision, due now but delayed a few months, on whether to go ahead in Britain with a demonstration commercial fast breeder reactor. The Minister responsible, Mr Anthony Wedgwood Benn, called for a public debate, and he's getting it. That there might be no "answer" seems to be distracting no one from the search.

The latest contribution comes in a joint publication from Friends of the Earth, the Council for the Protection of Rural England and the National Council of Civil Liberties called *Nuclear Prospects*. Subtitled "A Comment on the Individual, the State and Nuclear Power", it begins to fill a widening hole in the debate, namely a consideration of the social and political implications of nuclear power. The two authors have written what they call a speculative and highly conjectural paper explicitly with the fast breeder decision in mind.

The authors say a postulated commitment to the fast breeder, involving hundreds of tonnes of plutonium-enriched fuel, thousands of fuel shipments annually and some 100 reactors (50 of them breeders), would pose security problems even more enormous than those already caused by existing threats of theft and sabotage. Any attempt to overcome them would extend vastly the existing system of surveillance, policing and vetting inside the industry, and would provide justification for their further extension outside too. Civil liberties would be so threatened that it might be easier for even the Minister himself not to be answerable to the public.

Equally, their argument goes, electricity authorities wanting sites for nuclear stations could clash with local people who, demanding a say in what happens to land, attract wider anti-nuclear sentiment. That would threaten civil discord through direct action if people were unconvinced of the efficacy of parliamentary scrutiny and suspicious of the independence of the Nuclear Installations Inspectorate. Altogether the possible consequences make nuclear power an issue not to be argued simply at a technical level.

Certain obvious points are to be made in confronting these arguments. The security problems posed now by theft, terrorism, sabotage and bombs—even by existing stocks of plutonium—already raise serious questions about civil liberties. Countries cannot opt out of the risks unilaterally if neighbouring countries are "going plutonium". And the social consequences of an insufficiency of energy could be as dire as any caused through the use of fast breeders.

Nuclear Prospects acknowledges the first two of these points, quickly (perhaps too quickly) disposing of them. The authors wonder if civil society would adapt to standards of military security for the benefits of plutonium-based power; perhaps it might. They contend that there is "a world of difference" between exceptional precautions to combat terrorism and the steady erosion of rights threatened by the day-to-day use of plutonium; perhaps there is. But they barely address themselves to the third point, the very one that makes the first two interesting at all.

This may be because there isn't much discussion generally of the matter. Certainly if there is now any received truth in the "great debate" it is the premise that the need for energy will increase and go on increasing in a way that makes the fast breeder decision a decision that must be taken and taken soon. Voluminous documentary support exists for that view, and it cuts little ice at the moment to say that since 1971 no generating plant has been ordered (the companies concerned are reeling) because of simultaneous and seemingly unending inflation and recession.

But consider. Britain, some say, is suffering a long term decline to a subservient neocolonial status in Europe and the West; if so, her predicted energy problems (though not perhaps the world's) could arise even further into the future than presently projected. That is more grist to the mill of those already looking to a comparably financed effort in energy conservation and such alternatives as wave and solar energy to tide them over to a day when fusion or hydrogen might offer inexhaustible supplies of energy. These people imagine a nuclear interlude for Britain, not a nuclear future. Is this really a fantasy? The matter needs more discussion, not just by scientists; it cannot be divorced from a discussion of Britain's future when the cost is so gargantuan.

For consider again. Even those who acknowledge, in strictly energy terms and not with an eye to Britain's electricity industry, the need for a fast breeder, recognise that because of the resources involved the project could only succeed through international collaboration. For Britain, that means with either the French and Germans or with the Americans. The question of who will have her, assuming she can even afford to buy in, is not being discussed in public. But it makes a big difference to the value of any mere internal debate.

Perhaps it is unfair to criticise *Nuclear Prospects* on points that are beyond its intended brief; it certainly deserves praise for trying to fill a great gap in the debate. Mr Benn's decision is mostly symbolic. The debate is far from ritualistic. □



An open letter to the Health and Safety Executive

Michael Ashburner of the Department of Genetics at the University of Cambridge gives his view on the UK Health and Safety Executive's recent proposals for regulating genetic manipulation experiments

Dear Sirs,

The Health and Safety Executive is, I hope, engaging in that popular sport, kite flying. Worried, perhaps, by the mutant monsters so vividly portrayed on television every Saturday evening in *Dr Who*, you write, in your recent Consultative Document "Compulsory Notification of Proposed Genetic Experiments in the Manipulation of Microorganisms":

No person shall carry on any activity intended to alter or likely to alter the genetic constitution of *any microorganism* unless he has given to the Health and Safety Executive notice, in a form approved by the Executive for the purposes of these Regulations, of his *intention* to carry on that activity (Draft Regulation 2 of the Health and Safety (Genetic Manipulations) Regulations 1976). (my italics).

Two recent UK Government reports, those of the Working Parties chaired by Lord Ashby (Cmnd 5880; January 1975) and Professor Sir Robert Williams (Cmnd 6600; August 1976), have considered the problems posed by the recent advances in molecular biology that make it possible to introduce, in a form in which it may replicate and perhaps be transcribed and translated, DNA from "foreign" organisms (including man) into microbial hosts. Both reports considered that such experiments may lead to great social benefit but that they may be dangerous. In view of the potential dangers of some experiments of this type the Williams' Report drafted a Code of Practice to be adhered to when doing such work. This Code lays down "containment" procedures of varying levels of severity to be used for different types of experiment. In effect this will mean that because of the cost of such facilities, these experiments will be done in relatively few laboratories under the strictest supervision.

As you know, the scientific community (a pompous but useful term) is deeply divided concerning the problems that might arise from the use of these new techniques. However, the

"moderate" view is probably that the recommendations of the Williams Report (and their equivalents in the USA) are reasonable and, until we know more about the reality of the dangers, will have to be lived with. Your draft regulation is, I understand, an attempt to give the Code of Practice legal teeth and to place its administration in the hands of the Health and Safety Executive.

The Advisory Group recommended by Williams would play a subservient role to the Health and Safety Executive, despite the fact that this Group alone would include not only technically competent scientists but also "individuals able to take account of the interests of employees and the general public". A central feature of the Advisory Group is that it would "command the respect of the public as well as of the scientific community". If the advice that such a group gives can be overridden by you it would certainly lose this respect and be weakened as a result. The need for the Advisory Group is not simply, as you imply, so that the scientists would not suffer from "unnecessary delay". It is envisaged as taking on a far more important role, independently referring experimental protocols and serving as a channel by means of which new safety features can quickly reach those most directly concerned, in the laboratory.

Of course a weakness of the Williams report is that scant attention is paid to the problems that arise if such techniques come to be widespread in industry. It would make little sense if academic scientists submitted to an essentially voluntary control and industrial scientists were either not controlled or had to submit to a legal control. Accepting that some form of control is required and that this control applies equally to industry, universities and so on—a by no means universal view—the question is: how best should it be applied? Your answers to this question are your draft regulations. I

wish to point out that these regulations, especially regulation 2, are bad from three points of view.

- You remove the Advisory Group or any similar body from any central role in the dialogue that must exist between those who do the experiments and those who administer the laws under which they are done. With respect, sir, you do not have the standing in the scientific community required for this job.

- Instead of being content with covering just the "genetic engineering" experiments your draft regulation would control the *whole of microbial genetics*. You do this despite the lack of any evidence that such control is required to protect public health and safety. The consequences of wording the regulation in this way are very serious. It would make innovation in this important field of study very difficult indeed. Furthermore, the burden on a scientist communicating with you in advance the protocol of his every experiment, for no obvious reason, would be so great that you would lose the confidence and goodwill of the scientific community. If this were to happen the dangers might be very real since you rely upon this community to draw both real and potential hazards to your attention. Remember that it was the scientists who made possible the "genetic engineering" experiments who brought their concern to public attention (*Nature* **250**, 175). If a result of the public debate so actively encouraged by them is that scientists find their day to day activities encumbered with endless red tape, some may be reluctant to speak out quite so publicly in the future—and that could lead to a disaster that any subsequent legal process could do little to remedy. It would be a tragedy if certain types of scientific work were driven "underground" as a consequence of a ridiculous regulation.

- The third reason that the draft regulation 2 is bad is that it is unenforceable. A regulation that cannot be enforced would be evaded, at first perhaps not in ways that would present any danger to health or safety, but

eventually by falling into general disrepute and perhaps being ignored totally. It is unenforceable because you would not be able to assess the mountain of forms that would descend upon you, and you would have grave difficulties in drawing a legal line between micro- and "macroorganisms". To enforce it rigidly would also require you to halt many quite "normal" human activities—for example the examination or treatment of people with X-rays (surely *likely* to alter the genetic constitution of microorganisms to which we are hosts), and the treatment of crops with chemicals (which even if they do not alter the genetic constitution of individual microorganisms will

surely alter the constitution of populations of microorganisms). Finally, it makes a nonsense of the evolutionary process: populations of all microorganisms are continuously changing their genetic constitutions and have been doing so since, quite literally, the origin of life. I fear that no law will stop this vast activity.

It is just not good enough to expect scientists to identify techniques (and organisms?) which may be excluded from regulation because they do not present "any potential hazard". Not only is the range of organisms studied so wide, it is impossible to prove that any human activity is completely safe under any circumstances. Dangerous

activities, on the other hand, can usually be positively identified. Whether you list activities to be excluded from the regulations or just those to be included, the regulations will have to be continuously amended to take the results of research into account.

It would be far more effective to draft the regulations to include only those techniques of *known* danger or which scientists judge to be of potential danger, and actively to involve both the scientific community and others in both the assessment of these dangers and the administration of the law itself.

Yours sincerely,

MICHAEL ASHBURNER

An open reply from the Director of the Executive

John Locke, Director of the Health and Safety Executive, takes up the points in Michael Ashburner's letter

Dear Dr Ashburner,

I am glad to have this opportunity of commenting on the points raised by you.

The Government and the Health and Safety Commission have both accepted what you call the "moderate" view that the recommendations of the Williams Report are reasonable. This means that the carrying out of the techniques described in shorthand as "genetic engineering" should be permitted where they offer prospects of social benefit, provided that adequate steps are taken to protect workers in the laboratories and people outside those laboratories from harm. The Williams Committee recommended that in order to achieve this protection those using these techniques, whether in official laboratories or in universities or in industrial firms, should be required to notify their intentions and to seek the advice of the Advisory Group which is being set up by the Government.

The proposals for Regulations, circulated for comment by the Health and Safety Commission, are intended to establish this requirement to notify. The Commission does not believe that it would be right in a matter of this importance to leave people free to decide whether to abide by the recommendations of the Williams Committee. They believe that everybody should be placed

in the same position of being required to give this notification.

The draft Regulations provide for notification to the Health and Safety Executive because we are a statutory body whereas the Advisory Group will have no statutory status. But I really must protest at the suggestion that the Advisory Group would play a subservient role to the Health and Safety Executive. This is simply not the case. It will be for the Advisory Group to lay down the conditions which need to be observed to enable the experiments to proceed safely. Our aim will be to make sure that the Advisory Group is in fact consulted, and secondly to make sure that its recommendations are followed. Both steps are necessary if workers and the general public are to be adequately protected and this is a responsibility placed upon us by Parliament.

Nothing in the Regulations therefore will remove the Advisory Group from its central role in the dialogue between those who wish to carry out experimentation of this kind and those who are competent to advise on the nature of the precautions which need to be taken.

The main point which you raise is that the proposed Regulations are drawn too widely and would catch many activities which do not present special hazards and which were not

dealt with by the Williams Committee. This is certainly true. But the Williams Committee clearly found it very difficult to produce a precise description of the kind of work which they wanted reported. And it is no good at all saying that scientists shall notify their intention of carrying out certain types of work unless they are told pretty precisely what is to be covered.

We hope that as a result of comments on the proposed Regulations, we may be able to suggest a definition of what is to be notified, which, as you say, will cover all those types of work which could present special hazards and require special controls, and which will not be exposed to some of the kinds of objections raised in your note. I entirely agree that neither we nor the Advisory Group should be subjected to "mountains of forms". On the other hand, I am sure you would agree that we must not devise a definition of what is to be notified which would leave out certain types of work which might prove to present special hazards. It seems to us we must err, if we err at all, on the side of having rather more than we want notified rather than too little.

I hope therefore that all those concerned in this field will help us with devising a definition of the activities to be notified to the Advisory Group and to my Executive which will be sensible, workable, and capable of offering the protection to which workers and the general public are entitled.

Yours faithfully,

J. H. LOCKE

Europe's drought (1)

Under the weather

R. M. Morris and R. A. S. Ratcliffe of the Meteorological Office, Bracknell, discuss recent anomalies of the weather and the general atmospheric circulation

THE drastic reduction in rainfall over much of the British Isles from early 1975 represented a rather sudden acceleration of a trend which began at the end of the 1960s. The average annual England and Wales rainfall for 1971–75 was 826 mm, the lowest mean five-year value since the 1850s. There have, however, been six five-year periods since 1825 with annual averages less than 840 mm, so the recent five-year rainfall may be expected to occur once in about 25 years.

Rainfall in the period May 1975 to August 1976 was exceptionally low—in fact the driest such period in England and Wales since 1727, when records officially began. The rainfall total for this period is provisionally assessed at 760 mm compared with the next lowest value of 809 mm in the eighteenth century. Records are regarded as unreliable before about 1825 and if one excludes years before that date the next driest 16-month period starting in May was in 1933–34 with a total of 900 mm. It is difficult to calculate return periods of such an unusual event, but the standard formula suggests at best a once-in-500-year occurrence.

In England the period 23 June to 8 July, 1976, was also exceptionally warm. Mean daily maxima were some 10 °C above the normal values for the time of year and reached 32 °C in some places. Such an event is also unprecedented over the past 250 years. These high temperatures were partly due to the very dry ground, which meant that a smaller proportion of incoming solar radiation than usual was used for evaporating moisture; more was thus available for heating the ground and hence the atmosphere.

Broader perspective

Despite the severity of dryness in England it is important to view the anomalies in a broader spatial and temporal perspective. The distribution of rainfall over most of Europe for the period October 1975 to July 1976, expressed as a percentage of the long-term average (see map), shows that the area of less than 75% broadly extends southwest from Sweden, across the low countries and France to northern Spain, embracing much of England, Wales, eastern Scotland and also southern Germany. Less than 50% is confined to southern England

and northern France with minimum values near the coast of central southern England. On the other hand above average rainfall has occurred over Norway and extreme northern Scotland probably extending across Iceland, whilst similar or greater excesses are evident over western Russia, the Balkans and southern Italy.

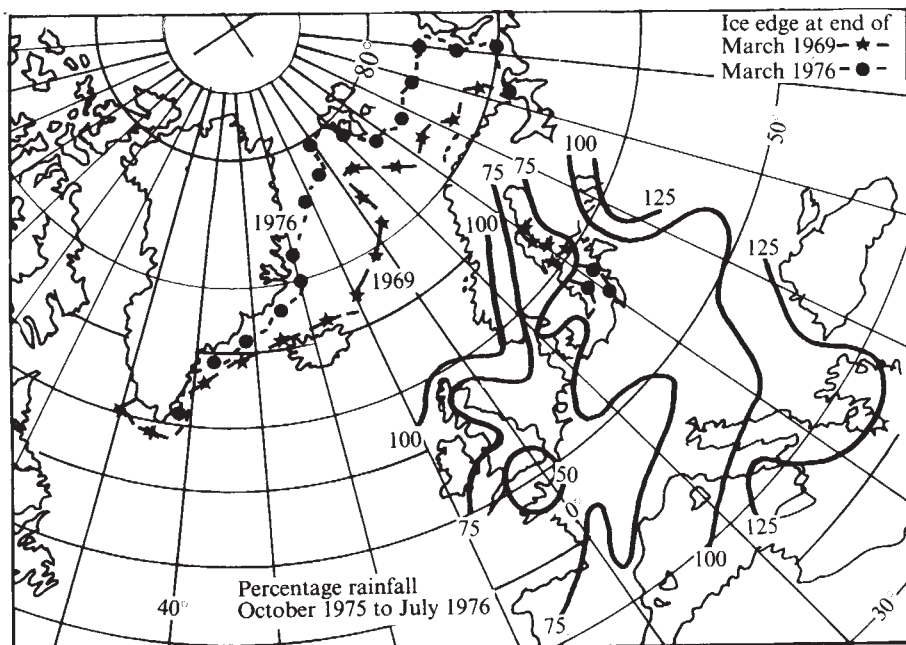
The distribution and evolution of rainfall anomalies on the scale shown is closely linked to the evolution of the major circulation systems in the tropospheric flow around the northern hemisphere. In particular the jetstreams (that is, zones of strong quasi-horizontal wind flow in the middle and upper troposphere) are usually closely associated with the zones of strong thermal contrast which separate regions of markedly different temperature and humidity characteristics. Jetstreams are intrinsically associated with weather because they represent a major cause of the upward and downward motion of the air in the middle layers which produces both extensive cloud systems and cloud-free regions in the circulation. The presence of a jetstream above a certain region usually implies changeable weather, with periods of rain alternating with periods of fine weather. Regions on the cold side of the jetstream (usually the northern side) tend to have a good deal of rain and showers; regions on the warm side tend to have a predominance of dry weather.

Within the past two years the location of the mean jetstream in the Atlantic–European sector of the circulation has shifted almost 1000 miles northwestwards in a coherent and continual manner. The second map illustrates the movement of July–August mean mid-tropospheric jetstream flow from 1974 through 1975 to 1976. Relatively small changes have occurred in the St Lawrence–Newfoundland area and also across the Mediterranean, and a major change has occurred between the northwest European seaboard and Iceland. To put matters in perspective, the mean summer position of the jetstream over the period 1951–70 was close to latitude 50°N across the Atlantic and extended rather weakly to the English Channel. With the jetstream in this position English summers tend to be rather cool and unsettled.

Jetstream shifted

During the winter of 1974–75 the main jetstream across Britain shifted to a position just northwest of the Hebrides. Apart from a temporary falter during the spring it maintained this position throughout most of 1975, moving a little further northwest during November and December. Despite some extremely vigorous cyclonic activity in the northwest Atlantic early in 1976, which actually encroached upon western parts of the British Isles during May, the mean jetstream clung tenaciously to a position between Iceland and Scotland.

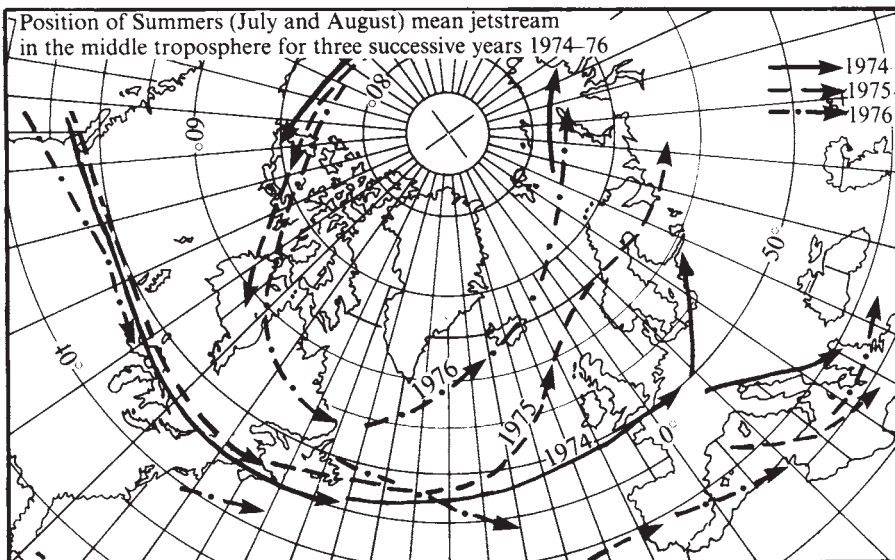
Following the recent summer the jetstream cannot extend further north in view of the proximity of the Arctic ice fields, which will inevitably extend southwards in the winter. The distribution of temperature gradient in the ocean–atmosphere system seems certain



to develop a new jetstream from the eastern USA across the Atlantic towards western Europe, thereby fundamentally changing the situation. Indeed this has already occurred and has been responsible for the recent heavy rain over much of the area worst affected by the drought.

The movement of the mean jetstream flow well to the north of the British Isles was the major cause of the marked reduction in rainfall, and the excesses of rain further north were clearly associated with an increase of jetstream activity not normally present in those latitudes. At the same time the influence of a jetstream upon rainfall anomalies is of limited geographical extent and such is the nature of the circulation that the predominance of cyclonic or anticyclonic regions in a particular region usually leads to the development of anomalous regimes in an adjacent region. The excess rainfall over the eastern and southern fringes of Europe is probably associated with this kind of circulation compensation.

The overall trend during the 1970s has been one of increased cyclonic activity in the Arctic basin, especially across the extreme north Atlantic and



Norwegian Sea, and increased anticyclonic conditions over western Europe. Such a circulation implies more frequent and stronger westerly winds than usual north of about 55° N in our sector. The increased westerly winds and cyclonic activity in the north have been associated with a marked recession northwards of the Arctic ice.

Up to 1969 Arctic ice to the north of Britain was extending southwards and reached its extreme southern position in March 1969 (see map). By March 1976 the area of open water in the Greenland-Spitzbergen area had increased by about 1.4 million square kilometres compared with March 1969. □

Europe's drought (2)

Water everywhere?

Haydon Richards of the Central Water Planning Unit assesses Europe's water resources

WATER is one of the natural resources with which Europe is better endowed than many other large areas of the world. This generalisation does, however, mask variations in geographical distribution of resources as compared with demands. The summer of 1976 has emphasised an extreme of variation. The driest 16-month period on record, from May 1975 to August 1976, has severely tested resources designed to provide a continuous water supply, but on the whole demands have been met and criteria used for design have proved to be sound. If living conditions are to be maintained or improved then demands have to be met for the four main sectors of public water supply, industry, agriculture and thermal power station cooling. The unusual conditions of 1976 have at least emphasised those areas where additional storage of water in some form needs to be made available in order to satisfy these demands and not impose severe restrictions on water use.

Estimating demand

Reasonably accurate estimates of

demand need to be made in order to plan development of suitable resources. Traditionally, trends over a number of years have determined the level of demand to be met at a particular time in the future, but more recent estimates have attempted to break down the components of the total demand. A good deal still remains to be done in this field. Publicity this summer has led to marked lowering of demand for public water supplies and for industry, amounting to at least 20% of normal demand in some areas. However, the knowledge that water is again abundant after a time may well reduce the attitude of care and thus increase demands to "normal" levels.

Apart from the demands to be met from the four major sectors the needs of ecology and amenity have to be borne in mind. Sufficient water must be left in rivers and lakes for aquatic life to continue, effluents to be suitably diluted, navigation to be possible and for fishermen and picnickers to enjoy their leisure. There must therefore be some idea of the quantity to be left in a natural watercourse to prevent un-

acceptable damage to the environment. This quantity can be determined only by agreement between all users of a particular river catchment and the criteria used may differ from one to the other, general amenity perhaps being over-riding in an upland or rural setting and effluent dilution in an industrial area.

Future projections in demand for water until the end of the century suggest high growth rates in excess of 200% in the Netherlands and at least 150% in Luxembourg, whereas in France demands may increase by little more than a quarter. Recent estimates indicate that Germany and the United Kingdom will need to increase use by 60 or 70%. Those countries with a high proportion of agricultural water demands locally, such as France and Italy, may have developed methods of meeting those demands locally, whereas the increased industrial demands in other countries have to be met by providing major new resources at some distance from the centres of demand.

Increasingly, demands for cooling water are being met from saline waters. Italy and Denmark benefit from long coastlines and use only a small proportion of freshwater for cooling in thermal power stations, although thermal pollution then becomes a problem. Some 30% of cooling tower water is evaporated, and this forms a consumptive use of water because it is lost as a resource. In the same way

water used for irrigation is almost totally lost as a water resource. Groundwater is important for meeting demands for potable water because it is usually of good quality, but only in France is it used to supply water for power station cooling. In Denmark, where there are no rivers of any size, nearly all water for public supplies, agriculture and industry is derived from groundwater storage, and in Belgium about three-quarters is so derived; in Ireland very little is used.

Water resources

Water resources are renewed annually from precipitation on the land surface, the effective rainfall being that which remains for surface runoff or infiltration to groundwater storage after evaporation and replenishment of soil moisture deficits.

Calculating average annual renewal of water resources provides an idea of the potential resource available in different countries. Ireland with its moist equable climate has approximately 32,000 litres a day available for each person from effective rainfall, whereas Belgium has about 3,000 litres a day; figures for other countries in north-west Europe lie between these.

The daily per capita consumption for all purposes in 1969 in Belgium was 119 litres and other countries range up to 280 litres. Even allowing for minimum flows to be maintained in rivers, therefore, nature still provides a substantial surplus of water which can be exploited to meet changing or increasing demands. Belgium's fresh water resources are found in three rivers and in extensive aquifers, and from the latter is obtained almost three-quarters of the public water supply. Regarding other European countries:

- On the low-lying Danish peninsula the small streams are fed by natural groundwater discharge. Some surface water is used to supplement the Copenhagen supply, but groundwater supplies nearly all the country's needs apart from thermal power plant cooling.

- Rainfall in France varies from about 850 mm per annum in the west to less than 500 mm near the Mediterranean. Below average snowfall in the Alps last winter slightly reduced some stream-flows during the summer of 1976. There are extensive aquifers in north east France and alluvial aquifers in the major river valleys.

- In West Germany there are also several rainfall zones, from the alpine south with high rain and snowfall, through the central highlands where the average annual rainfall is at least 1,000 mm, to the central lowland with an annual average of 730 mm. The flows of all the main rivers are fairly uniformly distributed throughout the year. Groundwater occurs mainly in the

flat alluvial tracts of the river valleys and beneath the northern plains, and provides well over half the public water supplies.

- Ireland is better endowed with water resources than most other countries in Europe, having a low population density and high rainfall which varies from 2,000 mm per annum on the west coast to little over 600 mm on parts of the east coast. There are several rivers and numerous lakes. Groundwater is widely used to supply farms but this source accounts for only about 7% of the total demand for potable water.

The Rhine is of first importance to water management in the Netherlands and it provides over 60% of the total, including power station cooling; groundwater provides more than a quarter of this total and the remainder is derived from the Meuse and small rivers. Navigation requirements also exercise control over water use because minimum levels have to be maintained to meet international agreements. Any open connection with the sea creates the possibility of an inflow of salt water and so substantial outflows are required in the areas below sea-level in the west of the country. Such control is linked to the general care taken to control salt water seepage into the polder areas. Potential resources from the Rhine are very large, but because of variations in the quality of the water management problems in the Netherlands are complex and involve storage in lakes, artificial recharge of sand dunes and the linking of existing sources.

There are marked geographical variations in rainfall in the United Kingdom; annual variations have been highlighted recently with rainfall in the year to August 1976 only 63% of the long term average over England and Wales. Groundwater provides 35% of public water supplies in England and Wales. Natural groundwater discharge provides 80% or more of the total annual flow of many rivers flowing from or over the major aquifer, the Chalk. Lesser percentages are contributed to streams from other aquifers.

Traditionally in the United Kingdom demands have been met in most areas by direct pipeline supply from upland reservoirs; reservoirs in the lower Thames valley are filled by pumping from the river. Along several rivers in England, water is used, returned as treated effluent and re-used lower down stream, and advances in the technology of water treatment in the last few decades has increased the possibility of using upland storage to regulate the flows of such rivers.

Water management

Both Ireland and Denmark tend to use a water resource and then ensure that the effluent is discharged after suitable

treatment to the sea. Countries with large inland industrial and urban areas such as France, Germany and the United Kingdom are faced with the problems of costly effluent treatment if the environment is to be protected, and particularly if water is to be re-used for further public, industrial or agricultural supply or amenity. In France, as in Italy, there are large numbers of small reservoirs in the hilly areas providing storage for farm irrigation, but this is not common elsewhere. Systems of control over agencies supplying water, managing rivers and dealing with effluent and sewage treatment vary greatly, but most countries have some form of statutory or voluntary means of tackling aspects of water development and use in defined catchments.

Direct supply of water from source to demand area will continue, but study of the relative costs of providing additional resources will probably produce different methods of using some existing sources. Schemes for the combined use of a number of sources can exploit their contrasting temporal characteristics, as for example when a supply from a river during winter is stopped during summer at times of low river flow, the summer supply being obtained by abstraction from groundwater storage which in turn is normally refilled by winter infiltration from rainfall. Such complex operations require suitable systems of data collection relating to quantity and quality of water in storage above and below ground, and the ability to forecast resources in store, changes in demand, operational problems and the likely results of any decision to use resources differently.

In spite of the uneven distribution of rainfall and of resources in rivers, lakes and aquifers there is sufficient water in the countries of north west Europe to satisfy foreseeable demands well into the next century. Care will be needed to ensure correct treatment of effluents, protection of the quality of surface water and groundwater and the maintenance of river flows for navigation and amenity. Additional storage is needed to meet shortages in certain areas, and by and large these have been emphasised during the dry period of the summer of 1976. This year has also shown that many of the major reservoirs have been designed so as to provide a large part of the required supply even through these unusual conditions. Groundwater storage has also helped by allowing abstraction at rates in excess of the long-term average renewal of storage, and the potential for even greater use during another severe dry period should be determined in necessary detail. No doubt methods of influencing demand patterns and modifying demands will be explored and developed. □

POPULATION

Will world population double?

Colin Norman looks at a new analysis of population trends throughout the world which has produced findings that are both encouraging and decidedly grim

THERE has been a dramatic slowdown in world population growth in the past five years as many countries, paced by China and the United States, have brought their birth rates down sharply. But death rates have risen steeply in some poor countries because crop failures and increasing pressure on agricultural resources and fisheries have claimed about two million lives. So says the study* of population trends conducted by Lester Brown and published by the Worldwatch Institute of which he is the President.

It paints a grisly picture of a world living from hand-to-mouth, with virtually no food reserves left to guard against poor harvests or other natural disasters, and with croplands, forests, grasslands and fisheries in many poor, densely populated regions close to breaking point. Brown concludes from the trends that "it's quite possible that we will never again see a doubling in world population growth, in spite of the fact that it is the standard rhetoric of UN officials", because for a great majority of countries, "a doubling of population will yield potentially unmanageable ecological, economic and political stresses".

Brown's principal conclusion is that "sometime near the beginning of this decade, the rate of world population growth reached an all time high and then began to subside". Though the total number of people in the world rose from 3,590 million in 1970 to 3,920 million in 1975, Brown estimates that the annual rate of increase has shrunk from 1.90% to 1.64%, a dramatic and, to some observers, surprising decline.

The bright side of the picture is that birth rates are falling in almost every country as more and more governments are reversing pro-natalist policies and instituting family planning programmes. Four European countries—East Germany, West Germany, Luxembourg and Austria—have already reached zero population growth and two others, Belgium and the United Kingdom, are expected to reach that point sometime this year. But by far the most dramatic reduction has

occurred in China and in some other East Asian countries.

With one-fifth of the world's people, China has a huge influence on overall population trends. According to Brown's estimates, China has just achieved "family planning's greatest success story", reducing the country's birth rate from a crippling 32 per 10,000 in 1970, to a more manageable 19 in 1975. Though hard and fast data for China are difficult to come by, Brown bases his estimate chiefly on figures supported by a variety of sources, on birth and death rates in political units in China, which he extrapolates to the entire population. His estimate for China's population growth is lower than one produced by the US Bureau of the Census a couple of years ago, but higher than a more recent estimate of the Agency for International Development.

But China isn't the only country to show a surprising reduction in its population growth. In some ways, the trend in the United States is equally striking. During the past five years, the growth rate in the US has declined by about a third, from 0.9 to about 0.6%, Brown estimates, in spite of the fact that children of the post-war baby boom have recently entered their prime reproductive years. The reasons include a drop in the marriage rate, increasing employment of women, and increasing female enrolment in graduate and professional schools.

And social trends elsewhere are equally significant. Mexico has recently completely reversed its pro-natalist policy and has instituted a strong family planning effort. And abortion laws have recently been liberalised in many countries, including predominantly Catholic nations such as France. In fact, Brown states that the proportion of the world's people living in countries where abortion is easy to obtain has increased from 38% to 64% in the past five years. "Few social changes have ever swept the world so quickly", he notes.

But in some parts of the world rising death rates have also played a part in reducing population growth. The grimest figures have come from the Indian sub-continent, particularly Bangladesh. Though Brown notes that few national leaders are eager to discuss rising death rates and good data are therefore difficult to obtain, he has pulled together data from a variety of sources which put into perspective the tragic impact of the Bangladesh war of independence from Pakistan and the

failure of the Indian harvest in 1972.

Brown bases his estimates for Bangladesh on meticulous records for death rates in the province of Matlab Bazar, maintained by the International Cholera Research Laboratory. The death rate in that province climbed from 15.3 in 1966-70 period to 21.4 in 1972. If the figure is extrapolated to the entire country, it suggests a nationwide increase in deaths from hunger of 427,000 that year. Similar extrapolations for 1974-75, when Bangladesh's rice crop was poor, suggest that the grim death toll was about 330,000.

Equally distressing figures emerge from extrapolations in India. In 1971 and early 1972, India had fed an estimated 8-10 million refugees from Bangladesh from its own grain reserves, but in late 1972 it was caught by two related disasters. First, the monsoon failed that year, severely reducing India's wheat harvest. And second, that same year, the Soviet Union imported 30 million tons of grain from the United States, thereby tying up most of the world's exportable wheat supplies. Official figures for death rates in India that year show an increase in Uttar Pradesh from 20.1 to 25.6, and equally steep jumps in two other provinces. Brown calculates that "in these three states alone, hunger claimed an estimated 829,000 lives". The figures in the Worldwatch report are the first authoritative estimates of the impact of crop failures on demographic trends in the region.

But Brown argues that death tolls from massive failure of crops and other disasters such as the Sahel drought are only one measure of the impact of overpopulation. Less obvious, but potentially more important is the fact that population growth is "simultaneously contributing to growth in food demand and to reduced food output". Overfishing is depleting fish stocks, he argues, and in many regions, overgrazing, deforestation and overploughing are leading to soil erosion, desert encroachment and the abandonment of crop lands. "It has been evident for some time that oceanic fisheries could collapse under the pressure of excessive demand. What is becoming equally clear is that land-based food systems can also give way under intense pressure", he argues.

For those reasons, he contends that it is difficult to believe that world population will ever double again, simply because few countries are capable of supporting double their present population.

Such realisations are beginning to show up in the implementation of family planning programmes. Nowhere is that development more striking than

**World Population Trends: Signs of Hope, Signs of Stress*, published by the Worldwatch Institute, 1776 Massachusetts Ave NW, Washington DC 20036. \$2.00.

in India, perhaps the nation hardest hit by hunger in the past few years. In the state of Maharashtra, the legislature has approved with only one dissenting voice a bill for compulsory sterilisation

of all males with three or more living children. Even if the measure is never implemented, the fact that such Draconian steps are being discussed is an important indicator of the serious-

ness with which India now regards the population problem. "Five years ago", Brown said last week, "compulsory sterilisation would not have been an acceptable topic for discussion in India".

NUCLEAR TRADE

Ford makes his move

With just five days to go before the Presidential election, Mr Ford last week announced long-awaited proposals designed to prevent countries which import nuclear technology from building atomic bombs. Colin Norman reports from Washington

MR FORD'S proposals, which have been extensively leaked during the past few weeks, represent some significant reversals of past Administration policy, and in some respects resemble less detailed suggestions made last May by his Democratic opponent Jimmy Carter. The crux of the new policy, outlined in a lengthy statement released by the White House, is that the United States should back away from its own plans to separate plutonium from spent nuclear fuel and recycle it as a reactor fuel. In addition, Mr Ford offered a raft of proposals for international co-operation in preventing the spread of nuclear weapons, including the following:

- He called upon all countries to refrain from selling reprocessing technology or uranium enrichment plants for at least three years. Already, West Germany has agreed to sell reprocessing and enrichment plants to Brazil, and France is committed to selling a reprocessing plant to Pakistan. Asked last week whether the Administration intends to enforce the policy in respect of those two deals, the Under Secretary of State for Economic Affairs, Charles W. Robinson, hedged by suggesting merely that "I think the statement that has been released by the President will be helpful in pursuing our interests in this matter".

- Mr Ford urged "new co-operative steps . . . to help assure that all nations have an adequate and reliable supply of energy for their needs". To that end he has asked the Secretary of State to initiate discussions with other suppliers to seek arrangements for co-ordinating fuel services. In particular, "these discussions will address ways to ensure against economic disadvantage to co-operating nations and to remove

any sources of competition which would undermine our common non-proliferation efforts". He also announced that the United States should increase its own enrichment capacity, particularly by expanding the government-owned plant at Portsmouth, Ohio. (The statement, it should be noted, was made in Cincinnati, Ohio.)

- Ford also stated that he has asked the Secretary of State to initiate international discussions aimed at establishing a new facility for storing plutonium and spent fuel from civil power stations, under the control of the International Atomic Energy Agency. He also pledged to place excess US plutonium in the facility once it is established.

- As for sanctions to deter nations from acquiring nuclear weapons, Ford stated that "any material violation of a nuclear safeguards agreement . . . must be universally judged to be an extremely serious affront to the world community, calling for the immediate imposition of drastic sanctions". In particular, he promised that the United States will, "at a minimum" respond to violation of any safeguards agreement to which the US is a party with "an immediate cutoff of our supply of nuclear fuel and cooperation to that nation".

In that regard, it should be noted that the atomic device exploded by India in 1974 was made from plutonium produced in a reactor which was moderated by heavy water supplied by the US under an agreement that it be used only for peaceful purposes. Does Mr Ford's threat to cut off fuel supplies therefore apply to India's apparent violation of its safeguards agreement? Mr Robinson would not commit himself last week, noting only that the United States has been negotiating with India to repurchase spent fuel from the Tarapur Atomic Plant, and that those negotiations "will be given new emphasis and support" by Ford's statement.

Asked later whether a second explosion by India would be sufficient to prompt a cutoff in aid, Robinson still refused to be drawn: "That obviously

would be viewed as a very serious matter . . . We must understand, however, that we are going back to an agreement concluded a number of years ago where our present concerns were not fully reflected in the contractual terms".

As for the United States' domestic nuclear programme, Ford said that he would speed up the effort to find a suitable method of disposing of radioactive wastes, and that he is directing the Secretary of State to initiate international discussions on the possibility of establishing centrally located, multinational waste repositories. His most important announcement, however, was that the US nuclear industry should no longer plan on reprocessing wastes and recycling plutonium.

A commercial reprocessing plant is already almost completed in Barnwell, South Carolina, and the nuclear industry has applied for a licence to begin recycling plutonium as a reactor fuel. Ford stated, however, that the United States "should no longer regard the reprocessing of nuclear fuel as a necessary and inevitable step." Reprocessing and recycling, he said, should only be permitted "if they are found to be consistent with our international objectives".

That leaves the Barnwell plant in a peculiar position since a decision to forego reprocessing would make it redundant before it even starts operating. One possibility, however, is that the Energy Research and Development Administration may seek Congressional approval to complete the plant and to operate it on an experimental basis to help evaluate the economics and the technological and safety problems associated with reprocessing. Ford's statement, in fact, said that he hopes to seek international participation in such an evaluation effort.

Finally, it should be noted that Ford's statement made no mention at all of an aspect of nuclear proliferation which many arms control experts see as central to the issue, namely, the controls which the superpowers are willing to place on their own weapons developments, particularly the testing of nuclear weapons. But those are touchy issues, and it was five days before the election. □

SWEDEN

● Less than a week after the formation of Sweden's new three-party government, Prime Minister Thorbjörn Fälldin's election promise to stop the insertion of fuel into the Barsebäck 2 nuclear reactor fell victim to the compromises said to be necessary for the survival of the coalition and insertion was allowed to go ahead. In a declaration of the government's programme, Fälldin said in effect that the government would stop the reactor going into operation unless its owners produce acceptable plans for the reprocessing of spent fuel and proof that radioactive waste will be stored in complete isolation from all life for eternity. The deadline for the presentation of these plans is October 1, 1977.

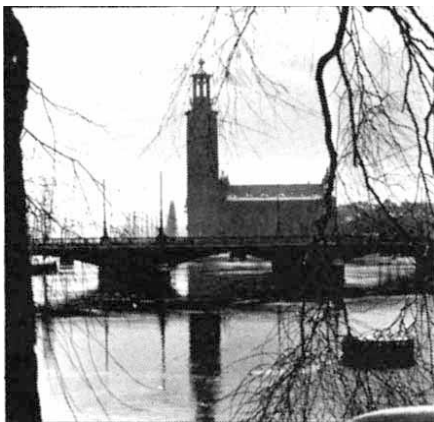
Politicians opposed to nuclear energy assert that the reactor's owners will not be able to come up with any proposals acceptable to the government. The nuclear industry is confident that it can. The effect is to throw open the entire question of whether the country's reactor plans are to be stopped, or even greatly slowed down: Sweden's construction workers are already seeking assurances about nuclear power stations now being built.

Fälldin also announced the formation of a commission which will evaluate the research done so far on the security and environmental aspects of nuclear power as well as the storage of highly radioactive waste. The commission is also to make suggestions for a new energy policy to be presented to parliament in 1978, until which time it seems that the futures of other reactors under construction will hang fire. It has at least been made clear that the twelfth in the planned series of thirteen reactors will not now be built at all, and this would presumably also apply to the thirteenth.

Meanwhile, in what has been dubbed as Operation Striptease, the findings of the Committee on Radioactive Waste have been attacked by academics and politicians who want to reveal its unwarranted optimism about the long-term storage of spent fuel. Action to endorse the committee's conclusions has come from only one source, a local government council which, amidst lively public debate, has voted to allow reprocessing and storage plants to be built near the Forsmark reactors already in operation.

● The hullabaloo over Barsebäck 2 has overshadowed a surprisingly favourable legal decision recently won

by one anti-nuclear power group. The Supreme Water Court has ordered the State Power Board to lower the level of radioactivity in the cooling water to be discharged from the third and fourth Ringhals reactors. In addition, the Court has directed the Board to pay about US\$22,000 in legal



costs to those who pressed the case, the environmentalist Björn Gillberg and Gunnar Michelson, a lawyer.

Following the Court's decision, the release of tritium must be lowered from 10,000 to 3,700 curies per reactor per year; of isotopes emitting beta and gamma rays, from 100 to 2–24 curies per reactor per year; and of isotopes emitting alpha rays, from 50 to zero curies per reactor per year. As the case of beta and gamma rays suggests, the new figures are not so much absolute limits as indications of what the Court thinks should be feasible, according to what is technically possible and economically reasonable. Its decision allows the Power Board to make very occasional releases of much greater amounts of radioactivity—but only when it may be technically impossible to avoid doing so. Any repeatedly greater releases would be investigated by the National Institute for Radiation Protection and could put the Board back in court for another trial.

Gillberg and Michelson lost that part of their case in which they had argued for a reduction in the volume of cooling water which is to be released into the sea. According to Gillberg, they are thinking of appealing on this issue to the Supreme Court. If they do, they will shore up the Prime Minister's creaking campaign to stop Sweden's nuclear power programme. The reactors' debut (planned for December 1977 and July 1979) would have to be postponed until the appeal had been heard and a judgment made, a process which would probably take two or three years.

● With nuclear fission such a controversial issue, the case for fusion could grow. Bo Lehnert, professor of plasma physics and fusion research at Stockholm's Royal Institute of Technology, says fusion would not involve the two problems normally associated with fission, namely radioactive waste products and reactor accidents; the radioactivity which neutrons would induce in the material surrounding the reaction could be diminished by using vanadium and its alloys in the wall material. He says that even if materials less suitable than these were used, the level of radioactivity and the corresponding biological hazards would be many orders of magnitude smaller than with a corresponding amount of energy produced by fission. Reactor accidents would also be less severe with fusion than with fission, says Lehnert, partly because no runaway nuclear reactions would be possible in an incident involving a fusion reactor.

Fusion power, then, is extremely attractive to the Swedes, as it is in many developed countries, and they have not been idle in their research. Their interest dates from the mid-1950s, Lehnert's group being financed mainly by the Swedish Atomic Research Council and the Bank of Sweden Tercentenary Fund. From 1976, however, it has joined with the team working under Hans Wilhelmsson, professor of electromagnetic field theory at Chalmers Institute of Technology, near Gothenburg, to form a fusion research unit associated with Euratom, which now shares the support of the unit with the Atomic Research Council. The immediate result of this has been a doubling of research funds, which amount this financial year to Skr4 million (about US\$900,000).

Lehnert is, however, dissatisfied with what he considers to be the narrowness of present fusion research. His general, and personal feeling—"controlled optimism for controlled fusion"—cannot give immediate comfort to anyone involved in the debate over fission reactors. Given a frontal attack on a wide variety of problems, including basic research, new ideas and modified old ideas, he believes that commercial fusion reactors may be "not quite unimaginable" before the year 2000. But with research going in current directions at its present level of finance, he forecasts a longer wait.

Wendy Barnaby

IN BRIEF

Ford statement attacked

President Ford was given a sharp rap over the knuckles last week by 10 Nobel prizewinners and one former Presidential science adviser for trying to make political capital out of the fact that the United States made a clean sweep of this year's Nobel prizes. Presenting the National Medal of Science to 15 American scientists last month, he took the opportunity to score a few political points, and without mentioning Jimmy Carter by name said that the Nobel sweep would "surely put to rest" suggestions that the United States had lost respect around the world. He then argued that there is a need to "bolster research and development to achieve national goals".

The remarks, and their implication that the Administration should share the credit for the Nobel awards, were attacked in a statement drafted by George Kistiakowsky, professor of Chemistry at Harvard and former science adviser to President Eisenhower, and signed by 10 Nobel prize-

winners including the winner of this year's award for Chemistry, William Lipscomb. The statement noted that "Nobel Prizes usually reflect work done over long periods of time. This year's prizes do not, therefore, reflect this year's strengths". The statement went on to argue that Mr Ford's budgets "have not been such as to encourage the growth of American science. The current appropriation for the National Science Foundation is actually 10% lower than it was in the year when Mr Ford took office." Other signatories were Kenneth Arrow, Julius Axelrod, David Baltimore, Owen Chamberlain, Carl Cori, Donald Glaser, Salvador Luria, E. M. Purcell and George Wald.

ESF optimism

At the conclusion of the European Science Foundation's second annual general assembly in Strasbourg last week the president of its Executive Council, Sir Brian Flowers, said he detected the emergence of a single European voice in science. The

assembly had accepted the findings of ad hoc committees on genetic manipulation and astronomy.

One material consequence is that a European committee will be established under the aegis of the ESF both to discuss recombinant DNA research and to keep under continuous review the guidelines covering it. The committee will consist of representatives of the various national advisory bodies concerned together with representatives from both the EMBO Standing Advisory Committee and the European Medical Research Councils.

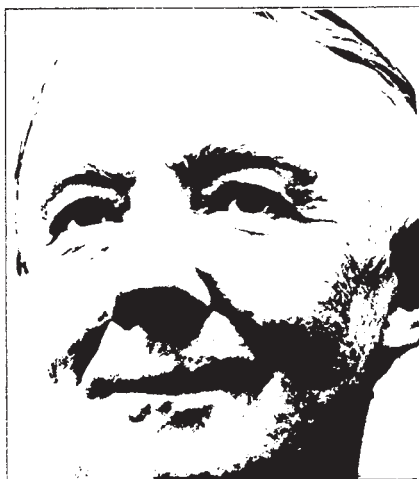
Acceptance of the astronomy committee's recommendations means that Europe's instrumental resources will soon be concentrated on a mountain ridge of La Palma in the Canary Islands. The first major step would involve moving the British Isaac Newton Telescope; Sweden's instruments now at Anacapri in Italy would follow, and a new Austrian telescope would come later. The Observatory itself would be Spanish.

At 10.30 a couple of Sundays ago I cut a tape across a footpath and led a crowd of 361 men, women and children on a sponsored walk around Grafham Water, one of the largest man-made lakes in southern England. Our purpose was to raise money for the Ouse Valley Wildlife Appeal to enable the Bedfordshire and Huntingdonshire Naturalists Trust, a voluntary conservation organisation of which I am president, to buy land for nature reserves. Almost all the walkers completed the modest circuit of ten miles, and so called upon the maximum generosity from those who had agreed to support the appeal, at rates ranging from a halfpenny to a pound a mile. My own efforts produced £56.20; the whole exercise raised more than £2,000. The Trust's last acquisition was Upwood Meadows, 14 acres of botanically interesting meadow probably unploughed since the Black Death; it cost us £5,000, so the walk paid off the debt on about five acres.

England is one of the most crowded areas in the world. The growing population, industry, affluence and increasing mobility are putting intense pressure on wild plants and animals and on the habitats which support them. Modern agriculture is changing the look of the countryside, and the wholesale removal of hedgerows and copses means that many birds, butterflies and flowers which were formerly common and widespread are difficult for the non-expert to find. Sites for scientific study, for

education and for ecological research are disappearing.

Fortunately there are in Britain official bodies like the Nature Conservancy Council and the Countryside

Walks for wildlife**KENNETH MELLANBY**

Commission which are charged with conserving wildlife and rural amenity, and this they do admirably to the limit of their meagre resources. It is encouraging that the Nature Conservancy Council has been able to designate an increasing number of outstanding National Nature Reserves in recent years, by purchase, lease or "nature reserve agreement". This last system is one by which liberally-minded landlords agree to restrict their activities so that the area will

retain its scientific importance. It is feared, however, that new legislation may compel some such landlords or their heirs to sell their property, and that the buyers may be less willing to give priority to a few rare orchids or to wildfowl.

It is therefore fortunate that official efforts are so often supplemented, and sometimes surpassed, by the many voluntary conservationist organisations in Britain. Critics may complain that conservationists are mainly a middle class elitist group, and this is to some extent true—and why not—but they are making great efforts to bring all sections of the population into their ranks.

Many of the reserves established by County Naturalists Trusts are conveniently situated to be used as open-air classrooms by local schools. When the pupils become involved in strenuous management exercises, such as removing unwanted scrub which is crowding out the more exciting plants, they may become converts to conservation. Even sponsored walks involve a new section of the population, and if, as at Grafham, these go through reserves with simple nature trails marking clearly some of the points of importance, the walkers' temporary interest may be made permanent. It is estimated that nearly five thousand of those who sponsored the walkers were not otherwise concerned with conservation; perhaps the success of the venture will turn their sympathy to real interest and support.

news and views

DNA sequences coding for more than one protein

from Benjamin Lewin

THE single strand DNA phages of *Escherichia coli* are amongst the smallest viruses known (only the RNA phages are appreciably smaller) and are therefore of special interest for the strategies evolved by their genomes to allow successful reproduction with such limited genetic information. In fact, a paradox in the organisation of their genomes has recently been suggested by the identification of a greater number of phage-coded protein products than it appears can be accommodated within the complexity of the genome. Both types of single strand DNA coliphage possess a circular genome, in the case of the icosahedral phages ϕ X174 and S13 fairly well characterised as about 5,500 bases, and for the filamentous phages f1, fd, M13, apparently somewhat larger.

A genome of 5,500 bases should have a maximum coding capacity equivalent to some 200,000 daltons of protein. But measurement of the proteins induced by infection with ϕ X174 identifies a total of nine species, with a combined weight approaching 250,000 (Benbow *et al.*, *J. Virol.*, **10**, 99; 1972; Godson, *J. molec. Biol.*, **57**, 541; 1971). Each of these protein products can be equated with one of the nine identified genes of the phage. In spite of some uncertainties about the sizes of two of the proteins, it is clear that their total mass is appreciably greater than is to be expected of the phage genome.

One mechanism utilised by the phage is to produce two proteins from one gene. Products of gene *A* can be identified with weights of about 60,000 and 35,000 daltons; and Linney and Hayashi (*Nature new Biol.*, **245**, 6; 1973) showed that the smaller protein represents the C-terminal part of the larger. Probably it results from an internal initiation of translation about halfway along the gene. The functional relationship between the two products is not clear.

A situation more difficult to resolve has been presented by the genes *D*, *E* and *J*, whose products have been identified as a protein of about 15,000 daltons needed for particle assembly, a protein of about 17,500 daltons needed for lysis,

and a particle component of about 9,000 daltons. The construction of a restriction fragment map for ϕ X174 suggested that there could not be room for all three genes in the part of the genome where their mutations lie. Restriction maps of ϕ X174 DNA have been developed with several different enzymes, including *Hind*II, *Hae*III and *Hpa*II; calibrating the sizes of the fragments with known standards generated a total length for the genome of 5,200–5,400 bases, slightly less than the previous estimates of 5,500 (Lee and Sinsheimer, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2882; 1974). By using a marker rescue technique, Weisbeek *et al.* (*Virology*, **72**, 61; 1976) recently were able to locate mutations on to *Alu*I, *Hind*II or *Hae*III fragments, and thus to calibrate the restriction map with the genetic map. This allowed the maximum possible size of each gene to be calculated, and in most instances this suggested that the protein products must be slightly smaller than the sizes previously estimated. But in the region where genes *D*, *E* and *J* lie, the sizes of the restriction fragments exclude the possibility that there might be three contiguous genes coding for proteins totalling more than 40,000 daltons. This seemed explicable only in terms of the postulate that mutants thought to identify *J* might in fact reside in *E*, eliminating the *J* gene from the map, but at the same time raising a question about the origin of the *J* protein and its relationship to the *E* protein.

In a striking demonstration of compact genetic organisation, Barrell, Air and Hutchison (page 34 of this issue of *Nature*) now resolve this problem. Using the "plus and minus" technique for DNA sequencing developed by Sanger and Coulson (*J. molec. Biol.*, **94**, 441; 1975), they have determined the sequence of genes *D*, *E* and *J*. (In fact, the entire sequence of ϕ X174 DNA has also been determined and is quoted as not more than 5,400 nucleotides, confirming the limits on genetic complexity previously established, and in good agreement with the total lengths of the restriction fragment

maps.) The sequence of the *D* gene was unequivocally correlated with its product by determining the sequence of the protein. The termination codon of gene *D* overlaps the initiation codon for gene *J* by one nucleotide: thus in the sequence TAATG, the first three bases provide the ochre codon terminating gene *D* while the last three bases provide the initiator for gene *J*. The following sequence can be defined as gene *J* by virtue of its equivalence with the amino acid sequence of a small protein isolated from the virion, which presumably corresponds to the product previously identified with *J*. However, the *am6* mutation that had been taken to define *J* genetically lies 179 nucleotides before the initiation codon for *J*, substantiating the suggestion of Weisbeek *et al.* (*op. cit.*) that it lies in another gene.

Where does gene *E* lie? Marker rescue experiments identified the location of two mutations, the classical mutation *am3* that has been taken to identify gene *E*, and the mutation *am6* that Weisbeek *et al.* suggested lies in *E* rather than *J*: both lie within the sequence equated within gene *D*. By determining the sequence surrounding the mutant amber codon, it is possible to define the phase in which gene *E* is read. This proved to be displaced one base to the right (that is in the direction of reading) from that of gene *D*. By scrutinising the sequence of gene *D* for an initiation codon (in association with a ribosome binding sequence) and a termination codon in this reading frame, it is possible to deduce that gene *E* resides within the latter part of gene *D*, and is read in a different phase (corresponding to a protein of 10,000 daltons, less than some previous estimates). Thus this sequence of DNA codes for two proteins.

How this situation may have evolved can be a subject only of speculation, but Barrell *et al.* suggest that probably gene *D* arose first, and that some event then led to the translation of part of it in a different phase, it so happening that the protein product possessed a high proportion of hydrophobic amino

acids that rendered it suitable to interact with the cell membrane, leading to the evolution of a lysis function. Of course, the evolutionary constraints upon the sequence representing both proteins must be very severe and it remains difficult to estimate how much scope there might be for variation in the sequences of the two overlapping functions. And, of course, overlapping expression must mean that all mutants in gene *E* also carry alterations in gene *D* (and *vice versa* within the region of overlap): while some of these mutations may affect one protein but not the other (due to third base degeneracy) it will be interesting in evolutionary terms to determine the general relationship between effects on *D* and *E* function. A possible utilisation of the overlap by the phage as a control is that one might expect ribosomes translating *D* to interfere with access to the initiation codon of *E*; this predicts that mutants causing termination early in *D* might have increased expression of *E*.

The complete sequence of the phage DNA will no doubt reveal whether the strategem of overlapping expression is used elsewhere in it, but the possibility is at least raised by the observation that the products of genes *A*, *B* and *C* have an estimated total molecular weight apparently somewhat greater than could be coded by this region of the genome.

What is the organisation of the genome of the filamentous phages? The genetic map constructed by Lyons and Zinder (*Virology*, **49**, 45; 1972) places the eight genes in a circular array; and with one reversal of order this was confirmed by the restriction maps calibrated with mutant locations in several laboratories (Van den Hondel *et al.*, *Eur. J. Biochem.*, **53**, 559; 1975; Seeburg and Schaller, *J. molec. Biol.*, **92**, 261; 1975; Horiuchi, Vovis and Zinder, *J. molec. Biol.*, **95**, 147; 1975). Proteins have been identified for six of the genes in two series of experiments; the results are in good agreement and suggest a total for the phage protein products of about 206,000 daltons (Model and Zinder, *J. molec. Biol.*, **83**, 231; 1974; Konings, Hulsebos and Van den Hondel, *J. Virol.*, **15**, 570; 1975). In addition, Model and Zinder identified a protein of 12,000 daltons, apparently phage coded but not yet equated with any gene.

Varying estimates have been taken for the size of the filamentous phage genome. Taking an estimate of 6,400 bases, and assuming that the genes *VI* and *VII* whose products are unidentified are about 250 bases each, led Van den Hondel *et al.* (*op. cit.*) to infer the presence of an unaccounted region of some 300 bases, which they located between genes *II* and *V* and suggested

might code for the protein of 12,000 daltons. Taking an estimate of 6,800 bases, and assuming that genes *VI* and *VII* are small, led Vovis, Horiuchi and Zinder (*J. Virol.*, **16**, 674; 1975) to suggest that there must be a considerable amount of intergenic material, which they placed between genes *IV* and *II*. The assumption that genes *VI* and *VII* are small is supported by the mapping of known mutations to restriction fragments, but as is clear from the location of unaccounted regions in different sites, there is some latitude in the construction of the map, resulting principally from the uncertainty as to where the ends of genes lie. (The restriction fragments of these phage genomes have not been sized with respect to outside standards but only relative to each other, which makes the construction of a physical map dependent upon the size assumed for the total genome.)

It has generally been assumed that the size of the filamentous phage genome is close to 15% greater than

that of ϕ X. This would suggest a length of not more than 6,200 bases, which is only 600 bases greater than the 5,600 bases required to code for the six proteins of identified origin. If there were no intergenic region, this would leave genes *VI* and *VII* able between them to code for some 22,000 daltons of protein. If an unidentified gene codes for the 12,000 dalton protein, then only 300 bases (or 11,000 daltons of coding capacity) remains for both these genes. In contrast to these calculations, Berkowitz and Day (*J. molec. Biol.*, **102**, 531; 1976) used biophysical means to measure a length for fd DNA of 5,740 (± 210) nucleotides. This would at best place a severe restriction on the length of DNA available to represent genes *VI* and *VII*; and if these genes should prove to code for larger protein products, or if the 12,000 dalton protein proves to represent a new gene, there would be the same paradox as seen with ϕ X174, with insufficient DNA to code for the phage products. $\square$

Lymphoid cell interactions in Japan

from Marc Feldmann

A Symposium entitled Cell Interactions in the Initiation and Regulation of the Immune Response, organised by Drs Yamamura and Hamashima, and sponsored by the International Union of Immunological Societies, was held in Kyoto, on September 1-4, 1976.

CELL interactions in the immune response were first recognised in 1966, with the work of Claman and his colleagues¹, and rapidly confirmed and extended by Miller², Davies³, Mitchison⁴, and others. At that time only interaction (or collaboration) between thymus derived (T) and bone marrow derived (B) lymphocytes was known, but the evidence of the following 10 years has shown that every immunological phenomenon involves interactions between several distinct cell types and their products. This symposium covered the whole spectrum of

modern cellular immunology and only a few of the aspects discussed can be reported here. Since lymphocyte heterogeneity is at the root of all these interactions recent developments in this field will take priority.

The many different surface antigens of lymphocytes have been exploited to purify different lymphocyte populations^{5,6}. The Thy-1 antigen which distinguished T from B cells⁷ is the most used. The three best characterised Ly (lymphocyte) alloantigens, Ly-1, Ly-2, and Ly-3, have been used by Cantor, Boyse and colleagues to define three subsets of T cells, bearing Ly-1,2,3, Ly-1, or Ly-2,3 antigens, which are present in decreasing proportions in the peripheral lymphoid pool.

H. Cantor (Harvard University) summarised recent work showing that helper T cells and cells involved in the mixed lymphocyte reaction (Ly-1) are distinct from cytotoxic T cells (Ly-2,3)⁷. The fact that suppressor T cells are also in the Ly-2,3 subset⁸, which is the smallest (5-10% of peripheral T cells) raised the interesting possibility that suppressor cells and cytotoxic cells may be identical, and the specificity of allo-aggressive suppressor cells like killer cells for the H-2K or D regions, rather than the I region was cited to support this view⁹. However, recent studies by Beverley *et al.*¹⁰ indicate that antigen specific suppressor cells (reactive to proteins) may be separated from cyto-

Correction

E. G. RICHARDS in an article in *News and Views* (*Nature*, **263**, 369; 1976) on September 30 entitled 'Complementary mispairs' inadvertently misnamed transitions and transversions on page 369. The word "transversions" on line 21 should be replaced by "transitions" and *vice versa* on line 24.

toxic T cells (reactive to H-2 antigens) by the use of anti-Ia antiserum, which lyses the former, and also by anti-Ly-1.1, which lyses cytotoxic cells of the CBA strain. On the other hand, the precursors of suppressor cells are usually not lysed by anti-Ia antiserum¹¹, and thus the two cell types could still be closely related.

It is now clear, however, that the Ly-1 and Ly-2 populations belong to different lines of differentiation and are not sequential stages in a single lineage¹². Cantor and his colleagues have shown that mice depleted of T cells (thymectomised, irradiated and bone-marrow-reconstituted mice or "B mice") and repopulated with Ly-1 T cells only were not able to perform any of the functions of Ly-2,3 cells, even many months later; and conversely "B mice" repopulated with Ly-2,3 cells could express cytotoxicity and suppression, but not other T cell functions.

There is great interest in the role of the major histocompatibility complex (MHC) in immune responses. One aspect which has attracted attention is the prohibition of cell interactions across allogeneic barriers where MHC differences are involved. This has been reported for T-B interactions by Katz and colleagues¹³, for T-macrophage interactions by Rosenthal and Shevach¹⁴ and Erb and Feldmann¹⁵, and for T cell killing, by Zinkernagel, Blanden and Doherty¹⁶. Several explanations have been suggested for these restrictions. One is that a molecule controlled by the MHC must be "shared" or recognised for successful interactions to occur¹³. An alternative suggestion is that mixing histoincompatible cells may induce active suppression^{9,17}. D. H. Katz (Harvard University), for example, found that suppressor cells are induced when F₁ thymocytes are primed in an irradiated parental mouse. These suppressor cells may explain the genetic restrictions on T-B collaboration found in earlier experiments¹³. Support for this possibility was discussed by H. Cantor and R. K. Gershon (Yale University) who have shown that removal of Ly-2,3 cell populations (which contain suppressor cells) permits the residual T cells to collaborate with histoincompatible B cells in the primary response *in vitro* to sheep red cells. Independently, Dutton and his colleagues¹⁷ have obtained similar results using anti-Ly antisera and cell separation techniques, indicating that allogeneic effects can suppress interactions between histoincompatible T and B cells. Thus, there is no doubt that suppression explains some examples of the failure of histoincompatible T and B cells to interact. Whether all such failures can be attributed to suppression is not yet clear.

EGF and viral transformation

from Robin A. Weiss

EPIDERMAL growth factor (EGF) was discovered by Stanley Cohen (*J. biol. Chem.*, **237**, 1555; 1962) when he was purifying nerve growth factor from the submaxillary salivary gland of male mice. Cohen noted that the factor stimulated the proliferation and differentiation of the epidermis, as reflected by precocious opening of the eyelids and eruption of the incisors in baby mice. Since that time it has been Cohen's remarkable achievement to purify and sequence murine EGF (Cohen and Savage, *Recent Progress in Hormone Research*, **30**, 551; 1974). It is a single-chain polypeptide containing 53 amino-acid residues with a molecular weight of 6,045. The molecule has been well conserved during evolution and human EGF extracted from urine shows only minor differences in amino-acid sequence to murine EGF. Recently it has become evident that EGF will stimulate proliferation of a variety of both epithelial and fibroblastic cells in culture. Its specific mitogenic action on non-proliferating cells in culture provides a much more convenient and quantitative assay than examining eyelid opening *in vivo*.

Last year Cohen's group showed that cells in culture which respond to the mitogenic action of EGF possess specific receptors for EGF on the cell surface (Carpenter, Lembach, Morrison and Cohen, *J. biol. Chem.*, **250**, 4297; 1975). EGF receptors were assayed by measuring the binding of ¹²⁵I-labelled EGF to the cell surface. Cells of many species bound EGF, but certain types, including a human lymphoblastoid line (NC-37) and rat cells transformed by mouse sarcoma virus

(KNRK) and by Rous sarcoma virus (XC) apparently lacked EGF receptors. Reduction in the binding of other hormones to transformed cells has also been reported, for example, in a recent study of insulin receptors on BALB/3T3 cells by Thomopoulos, Roth, Lovelace and Pastan (*Cell*, **8**, 417; 1976). The binding of insulin varied with the level of proliferation in cell population and the reduction seen in transformed clones was not specific to the transforming agent. However, the presence of EGF receptors appears to be more specific. In this issue of *Nature* (page 26), Cohen, in collaboration with Todaro and De Larco, reports additional data on the binding of EGF to the same cell types transformed by different tumour viruses. For example, the A31 clone of mouse BALB/3T3 cells was transformed by SV40, two strains of mouse sarcoma virus (MSV) and Rous sarcoma virus (RSV). Only the MSV-transformed subclones failed to bind EGF. This remarkable specificity of binding was true for transformed subclones of other types of cell such as Swiss 3T3, NRK and Mink lung. In each case, only the subclones transformed by MSV or by feline sarcoma virus failed to bind EGF.

Solely on the basis of the specific failure to bind EGF, the authors postulate that MSV might transform cells by coding for an EGF-like molecule in part of its sarcoma-specific genetic sequences. This EGF analogue would then block the receptors and stimulate the cells to grow. This will hardly provide the whole explanation of viral oncogenesis, but it can be tested.

It now seems clear from the reports of several workers that suppression is mediated by products of the I region. T cell suppression in mice, for example, is mediated by an antigen-specific T cell factor with a molecular weight of about 50,000 and which does not operate across H-2 barriers^{18,19} (T. Tada, Chiba University). This factor is absorbed by antisera reactive to products of the I-J subregion, which also seems to code for the receptor for the suppressive factor. The cells carrying the receptors (sometimes known in this context as acceptors) were found not to be helper T cells, but nylon-wool-adherent T cells which could be killed by anti-I-J serum and complement^{18,19}. Allotype specific suppressor T cells can also be killed by antiserum containing antibody to I-J region products²⁰⁻²² (L. Herzenberg

and D. B. Murphy, Stanford University).

Two generalisations seem to be possible on the basis of this and other work. First, that the I region is functionally specialised, the I-J subregion being involved in suppression and the I-A subregion in macrophage or T-cell helper functions. Second, the products of the I region act on target cells through receptors controlled by the same I subregion^{23,24}.

M. Taniguchi (Chiba University) discussed studies performed with Tada on a factor obtained from lysed antigen-primed spleen or thymus cells which augmented IgG antibody responses, provided that factor donor and recipient (in culture) were identical in the I-A region. This factor seems to be different from the helper factor described by

Taussig and Munro²³ which does not require I-A identity. Furthermore, the material isolated by Taniguchi and Tada unlike that of Munro and Taussig does not induce immune responses in the absence of other T cells, suggesting that it is an amplifier factor, rather than a helper factor which replaces T cell function. Thus, there are now four examples of I region factors each of which acts through a receptor on another cell type, but which is controlled by the same I subregion¹⁹⁻²⁴. The presence of (at least) three pairs of genes for factors which augment responses and their receptor each detected by different techniques in different assay systems, but all controlled by I-A, leads to the obvious question as to whether the factors described by Taussig²³, Erb²⁴, and Taniguchi¹⁹, are really distinct members of a 'family' of factors, or whether they are the same, but seem different because of the assays used. T cell suppression is also under control of non-H-2 genes. This was reported by Tada on the basis of the inability of cells from the A/J mouse strain to make suppressor factor, and of cells of the B10 congenic strains to accept suppressor factor^{18,19}.

The mechanism of suppression of the immune response by T cells is not yet known in detail. Tada noted that suppression involves an intermediate cell, which is involved in suppressing helper cells. J. F. A. P. Miller (Walter and Eliza Hall Institute, Melbourne) reported that suppressor cells induced during tolerance induction cannot suppress on their own but require the participation of adherent cells²⁶. E. Diener (University of Alberta, Edmonton) also described studies in which adherent cells were involved in the mediation of tolerance²⁷. This suggests that adherent cells (perhaps T cells, or cells of the macrophage/monocyte series) are involved in the effector phase of suppression. Clearly the relationship of this cell, which may be of hitherto unknown type, to known cell types, is an important question for classification.

Miller's work emphasised that we do not yet know how many types of lymphocyte there are. He reported that cells which suppress induction of the delayed hypersensitivity response in mice are Ly-1 cells, unlike the Ly-2,3 suppressor cells which inhibit antibody response. This difference could be explained if there is T-T interaction in induction of the suppressor cells of the delayed hypersensitivity response with Ly-1 cells stimulating (*in vivo*) Ly-2,3 precursors of suppressor cells to become active suppressor cells. Such a T-T interaction in the induction of suppressor cells has been reported previously²⁸. T. Hamaoka (Osaka Uni-

versity, Japan) reported studies which confirmed the T-T interaction concept, using hapten reactive T cells.

The field of cell interactions in the immune response has clearly expanded considerably over the past 10 years, and it is predictable that the next 10 years will see a deepening awareness of the complexity and richness of the cell interactions and suppressive effects which regulate and integrate immune response.

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Gravity experiments

from Malcolm MacCallum

STANDARD introductory courses in general relativity give an account of the "three crucial tests" discussed by Einstein in his famous 1916 paper: the gravitational redshift, the bending of light-rays and the advance of perihelia of planets, especially Mercury. These established the superiority of Einstein's theory to the others of the day, but many more theories have been invented in the intervening 60 yr. Only since 1970 have new experimental tests become available.

The classic tests are of course still actively pursued. The Eötvös experiment, which checks that the gravitational acceleration of laboratory bodies is independent of their composition, was improved to one part in 10^{12} by

V. A. Braginsky and V. I. Panov in 1971. Gravitational redshift measurements, both astronomical and terrestrial, which can be interpreted as a test of the universality of free-fall (given energy conservation), are accurate to a few per cent. Clocks in spacecraft should improve on this, and a satellite experiment is already in progress.

It is difficult to carry out the second "crucial test" with visible light. The Texas team which recently reported on observations made in Mauretania in 1973 (*Astr. J.*, **81**, 452; 1976) quote an error of about 12%. Measurements of the deflection of radio waves are more accurate. The most recent result, announced by E. B. Fomalont and R. A. Sramek (*Phys. Rev. Lett.*, **36**, 1475; 1976) quotes an error of about 1%. This limit cannot really be improved without detailed knowledge of the conditions in the solar wind plasma through which the waves pass.

The perihelion measurements have been checked by radar to about 1%. Their meaning was thrown into question 9 yr ago when R. H. Dicke and H. M. Goldenberg observed an apparent oblateness of the Sun which suggested a solar quadrupole moment large enough to produce a significant part of the effect, but H. A. Hill and R. T. Stebbins (*Astrophys. J.*, **200**, 271; 1975) found a much smaller corrected value. All three of the "crucial tests" are thus in good agreement with Einstein's theory.

It has been known for some time that further effects are potentially observable. The first of these actually to be measured was the "fourth test of general relativity", the time-delay of electromagnetic waves passing near the Sun. This has been measured by radar echoes from Mercury and Venus and more recently, by J. D. Anderson *et al.* (*Astrophys. J.*, **200**, 221; 1975), using the Mariner 6 and 7 spacecraft, to about 3%.

Possible experiments on gravity have been extensively examined since 1968 by K. D. Nordtvedt at Montana State University and by a group at California Institute of Technology led by K. S. Thorne. The simplest theories to analyse are those in which the influence of gravity on matter is completely described by a (Riemannian) metric, as it is in general relativity. These theories differ in how the matter generates the metric. The analysis proceeds by computing all possible effects arising from the lowest order corrections to Newtonian theory. The most general form of this "parametrised post-Newtonian framework", due to C. M. Will (*Astrophys. J.*, **185**, 31; 1973) involves 10 parameters dependent on the particular theory considered. Theories which do not fit within this framework are more difficult to analyse systematically, but several of them have already been

shown to be in conflict with observation. The programme has drawn attention to the possibility of new types of experiment and new experimental results on two of these have been announced recently.

One of them is the Nordtvedt effect, which arises if gravitational energy does not obey the equivalence of inertial and passive gravitational mass. Since the gravitational energy content of laboratory bodies is extremely small, this is not tested by the Eötvös experiments. Laser-ranging of the Moon has been carried out since 1969 to search for the effect. The results obtained and analysed by a team of seventeen from nine American institutions (J. G. Williams *et al.*, *Phys. Rev. Lett.*, **36**, 551; 1976) and independently analysed by I. I. Shapiro, C. C. Counselman III and R. W. King (*Phys. Rev. Lett.*, **36**, 555; 1976) show that the equivalence principle for gravitational energy is true to about 3% accuracy.

The other new result concerns variations in gravimeter readings, like those produced by Newtonian tides of the solid Earth. As shown by C. M. Will, these variations could contain post-Newtonian contributions dependent on lunar, solar and sidereal periods with 12-h, 24-h and annual oscillation. Most of them are too small to be detectable. Nevertheless, existing data enabled Will to place strong limits on the influence of any preferred reference frame (essentially an absolute rest frame), and to show, for example, that Whitehead's action-at-a-distance theory of gravity, which had lasted 50 yr, predicted effects 200 times larger than experiment allowed. Effects of these types have been further restricted by new data produced by R. J. Warburton and J. M. Goodkind of the University of California at San Diego (*Astrophys. J.*, **208**, 881; 1976) using a superconducting gravimeter. The main uncertainty lies in the calculation of the Newtonian effects, especially those arising from the shifting weight of the oceans.

All the experiments mentioned above, together with those also analysed by Will on the equivalence of active and passive gravitational mass and on the Earth's rotation rate, are in agreement with the predictions of general relativity. The only discrepant result is the time-dependence of the gravitational constant given by T. C. van Flandern (*Mon. Not. R. ast. Soc.*, **170**, 333; 1975) but even this differs from zero only by twice the quoted error. Further results from these measurements and from, for example, the precession of gyroscopes in satellites, can be hoped for in the next decade.

One might thus think that Einstein's theory was proved. Certainly the work

of the past 5 yr has severely damaged the credibility of those rival theories available in 1970. Unfortunately, any finite number of effects can be fitted by a sufficiently complicated theory. Indeed, all the general-relativistic predictions for the post-Newtonian effects can be replicated to arbitrary accuracy or even exactly by suitably constructed alternative theories. Aesthetic or philosophical motives will therefore continue to play a part in the widespread faith in Einstein's theory, even if all tests verify its predictions. □

Histone gene organisation

by Eleanor Lawrence

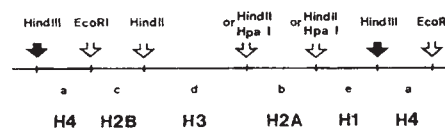
OVER the past few months a detailed picture of histone gene organisation at the level of the repeating unit has begun to emerge, based on the work of several groups who have approached the problem with a variety of different techniques.

In a recent set of papers (Gross, Probst, Schaffner and Birnstiel, *Cell*, **8**, 455; 1976; Schaffner, Gross, Telford, and Birnstiel, *Cell*, **8**, 471; 1976; Gross, Schaffner, Telford and Birnstiel, *Cell*, **8**, 479; 1976) Max Birnstiel and his colleagues have ordered the individual genes within the histone repeat unit of sea urchin DNA—in this case *Psammechinus miliaris*. They have ordered the genes by hybridising restriction endonuclease-generated fragments of enriched *Psammechinus* histone DNA with highly-purified individual labelled histone mRNAs isolated from the labelled 9S polyribosomal RNA from a closely related sea urchin *Paracentrotus lividus*, each of which has been assigned to one of the five histones H1, H2A, H2B, H3 and H4, by comparison of the *in vitro* translation products with *Paracentrotus* histones labelled *in vivo*.

A major difficulty in previous attempts to order the individual coding sequences in this way, has been the separation and identification of mRNAs coding for histones H2A, H2B and H3. By a careful choice of conditions, based on a study of the complex behaviour of *Paracentrotus* histone mRNA on electrophoresis under various denaturing conditions, Gross *et al.* were able to obtain five distinct mRNAs of sufficient purity that on translation in a wheat germ cell-free system four of the mRNAs could be characterised as coding mainly for a single protein which could then be identified as one of the histones by comparison with ³H-leucine and ³H-lysine labelled histones produced *in vivo*.

From digestion of enriched histone

DNA by a set of four restriction enzymes a detailed restriction map of the complete unit was constructed. Using combinations of restriction enzymes the 6 kb complete repeat unit was subdivided into five fragments. Each fragment hybridised with only one of the purified mRNA fractions and so by collating the restriction map and the hybridisation data, the individual coding sequences could be ordered within the repeat unit as shown below.



The polarity of the *Psammechinus* histone genes has also been determined. Limited resection by lambda exonuclease (which digests from the 5' end only) of the complete repeat units obtained after restriction with *HindIII* or *EcoRI*, and subsequent hybridisation to H4 mRNA showed that the polarity of the H4 gene was 5' H2B→H4→H1 3'.

Further experiments of this and other groups have shown that the histone coding sequences all lie on the same DNA strand, and so it follows that all the coding sequences have the same polarity, and transcription will proceed in the direction H4→H2B→H3→H2A→H1.

Groups at Stanford and CalTech have since reported a similar order for the histone genes in another sea urchin—*Strongylocentrotus purpuratus* (Cohn, Lowry and Kedes, *Cell*, **9**, 147; 1976). In this case pure histone DNA was isolated by cloning the two *EcoRI* restriction fragments of ~2.2 and ~4.8 kb, which make up the complete '7 kb' histone repeat unit in plasmids pSp17 and pSp2, respectively. Further dissection with restriction endonucleases enabled a detailed restriction map of the repeat unit to be constructed and fragments corresponding to various classes of histone mRNA were identified by hybridisation. An earlier tentative assignment of these mRNAs to individual histones (see *News and Views*, **259**, 361; 1976) has required revision, and the order of the genes has now been determined by direct sequencing of portions of the plasmid-cloned DNA fragments corresponding to the different mRNA classes (Sures, Maxam, Cohn, and Kedes, unpublished) and matching them with the known histone amino acid sequences. The genes appear to be ordered in exactly the same sequence as those in *Psammechinus*.

A detailed quantitative assessment of the relative positions of the coding and spacer regions is provided in the

accompanying paper by Wu *et al.* (Wu, Holmes, Davidson, Cohn and Kedes, *Cell*, 9, 163; 1976), who have hybridised total histone mRNA to single-stranded DNA from the histone plasmids pSp2 and pSp17.

Since the mRNAs hybridise only to the complementary coding sequences, the arrangement of coding and non-coding regions can be seen directly in the electron microscope after a novel treatment with T4 gene 32 protein which preferentially stains the single-stranded regions of the histone plasmid DNA-histone mRNA hybrids (Wu and Davidson, *Proc. natn. Acad. Sci. U.S.A.*, 72, 4506; 1976). As expected, they found three RNA:DNA hybrid regions on pSp2 and two on pSp17 accounting for the five histone genes, interspersed with spacer DNA—definitive proof that each gene is only repeated once in each repeat unit. Direct measurements allow them to independently assign most of the duplex regions to specific histone genes, constructing a map which agrees with those obtained by other methods.

Portmann, Schaffner and Birnstiel

(page 31 of this issue of *Nature*) have also directly demonstrated by electron microscopy five coding regions interspersed with spacer DNA in the *Psammechinus* repeat unit DNA cloned in phage lambda, by making use of the fact that the AT-rich DNA of the spacer regions denatures at a lower temperature than the GC-rich coding sequences forming open loops along the DNA. The GC-rich regions correspond well in size to the individual histone protein sequences.

Detailed sequencing of the repeat unit at the nucleotide level is now underway, made possible by the ability to obtain pure DNA fragments by cloning in phage or plasmids. Before too long, nucleotide sequencing may well have identified any regions of homology between the non-transcribed DNA in these and other genes, or between the histone spacer sequences themselves. Preliminary evidence (Cohn *et al.*, *Cell*, *op. cit.*), indicates that most of the histone spacer sequences are unlikely to be shared with other genes, and that there is also little homology between the individual spacer sequences. □

keeping population levels steady.

Hogarth concludes with speculation on the causes of extinction of dragons: despite persistent accounts of dragons and similar animals even in the present century, the typical mediaeval dragon was certainly extinct by the late 18th century. One contributing factor was commercial over-exploitation, primarily for pharmacological purposes. Only once was conservation legislation passed to protect dragons. This was in Rhodes, in 1345, when the king forbade any knight to attempt to slay a local dragon (although Hogarth conjectures that this edict stemmed from concern for the knights, not the dragon). If we accept the notion that dragons were extreme K-selected animals, then their rapid extinction under the diverse pressures exerted by man is not surprising (see for example, *Nature*, 257, 737–738; 1975).

Hogarth's article is undoubtedly seminal, but I find it in some respects excessively uncritical. In discussing the evolution of dragons, and other "related species such as the cockatrice and griffon". Hogarth suggests they "probably originated as a distinct group only 5,000 years ago". Quite apart from the inherent implausibility of this statement, it is well to begin by getting clear the morphological details of the animals loosely grouped together here. These can be obtained from bestiaries, or from any heraldry text. Setting aside relatively minor differences, such as whether the feet have talons or claws, or whether the head has teeth or a beak, the basic difference is that the griffon and the canonical dragon are six-limbed (four legs, two wings), whereas the wyvern and cockatrice are four-limbed (two legs, two wings).

This is an absolutely fundamental distinction. One of the most conservative features of vertebrate evolution is the tetrapod morphology: this may be seen in any museum exhibit of the

The ecology of dragons

from Robert M. May

ALTHOUGH much studied in earlier times, dragons and their ilk have been largely neglected in the recent upsurge of interest in animal ecology and behaviour. An article by Hogarth (*Bull. Brit. ecol. Soc.*, 7[2], 2–5; 1976) seeks to remedy this neglect.

In view of the lack of contemporary observational evidence, Hogarth necessarily relies on a survey of earlier sources. Most of these are from the 17th and early 18th century, an age when scientific curiosity was flowering. Later publications are increasingly sceptical, although Hogarth notes published doubts on the existence of dragons as early as Caxton's (1481) *Mirror of the World*.

Dragons appear to have been both omnivorous and voracious. Different records testify to their diet having been highly variable in both composition and quality: one dragon ate two sheep every day, and another which was kept captive by Pope St Sylvester consumed 6,000 people daily. The population density was also highly variable (presumably in a way which correlated with the per capita food requirements): "in England, indigenous dragons were solitary and it is doubtful whether the resident population averaged more than a few dozen, although occasional migrant flocks of up to 400 were seen: in India, by contrast, the marshes and mountains were described as being 'full' of dragons". Estimates of their life table parameters are scrappy, but

there seems to be general agreement on a typical lifespan of the order of 10^2 – 10^4 years.

The sexual display behaviour of dragons includes at least one remarkable and unparalleled manifestation, recorded by an 18th century author: "Dragons, being incited to lust through the Heat of the Season, did frequently, as they flew through the Air, Spermatise in the Wells and Fountains". This may be conjectured to have had adaptive value in reducing intrinsic fecundity. Such long-lived beasts would seem to have been at the extreme K-selected end of the r–K continuum, and would therefore be likely to exhibit behaviour which had the effect of

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500,000,000 years of evolution from lobe-finned fishes through amphibians and reptiles to birds and mammals. This underlying conservatism in skeletal structure, despite great variation in outward form and function, probably reflects the relative ease of modification of genes which govern timing in development, as opposed to those governing basic structure (see for example, King and Wilson, *Science*, **188**, 107-116; 1975). The wyvern and cockatrice have this basic vertebrate tetrapod morphology, but the six-limbed dragon and griffon do not. The probable ancestry of these latter two, as an entirely separate group, therefore dates back at least to the Devonian. This basic distinction applies to other now-extinct beasts: despite superficial similarities, unicorns belong with the familiar tetrapods, but the pegasus belongs with the six-limbed dragon-griffon vertebrate phylum, as do centaurs. Some angels (the humanoid-plus-wings kind) also belong in this phylum, but in view of the bewildering complications of angel morphology (once one includes cherubim, seraphim, and so on: see Davidson, *Dictionary of Angels: Including the Fallen Angels*, Free Press, 1967), this point is best not pursued.

In brief, wyvern and cockatrice can be envisaged as radiations from the basic vertebrate theme. But dragons, griffons, centaurs and angels belong to an entirely different lineage, the evolutionary history of which is shrouded in mystery.

The loose association of these two fundamentally different groups provides a striking example of the pre-Darwinian tendency to regard each species as a separate act of creation, rather than to trace logical phylogenetic relationships.

On the other hand, grouping together dragons, wyverns and the like is understandable in the light of the similarities of their ecology, behaviour and superficial appearance. They provide a dramatic example of evolutionary convergence, in the face of phylogenetic differences at least 400,000,000 years old. Such convergence implies some very tight evolutionary constraint somewhere in the "dragon" niche, a constraint hardly hinted at in Hogarth's account of their highly generalist diet and behaviour. This constraint may lie in the tendency exhibited by most dragons of record to be obsessive custodians of hordes of gold.

I conclude with the time-worn call for further research, modified by the highly contemporary remark that (if the above speculation is correct) such research may yield the literally golden fruits that grant-giving agencies increasingly desire. □

Primate behaviour and ecology

The Sixth Congress of the International Primatological Society was held at Cambridge on August 23-27, 1976. Aspects of the conference dealing with behaviour and ecology are discussed below.

from F. P. G. Aldrich-Blake and
Miranda Robertson

THE theoretical thrust of much primate work in the late sixties and early seventies was loosely socio-ecological; features of social organisation such as group size and composition were related to broad ecological categories of habitat such as 'forest' or 'savanna'. While this approach sought an evolutionary explanation of primate societies, it was insufficiently precise both in its treatment of causal mechanisms of social change and in its measurement of critical ecological variables. Recent work has placed a greater emphasis on the adaptive strategies of individuals in their dealings with society and the environment.

Notable in this respect was a paper by R. M. Seyfarth, D. L. Cheney and R. A. Hinde (University of Cambridge), which sought to provide a conceptual framework within which to interpret inter-individual behaviour. Primate societies, they pointed out, can be analysed at three different levels: interactions between individuals; the long-term relations to which interactions give rise; and the structure resulting from those relationships. While patterns of interaction between individuals are often apparently complex, they may be governed by relatively simple principles. For example, networks of social grooming among adult females of four species of primate had many features in common, despite being drawn from groups of differing size and degree of genetic relatedness. Computer simulation showed that these features could be accounted for by a preference for females of high rank as grooming partners and competition for access to these preferred females. Interactions between individuals have long-term effects on their relationships: grooming partners are more likely to form coalitions during aggressive encounters than are pairs that have had fewer friendly interactions in the past. Thus the optimum individual strategy should be to maximise the benefit derived from others by maximising the time spent interacting with animals of high rank. A similar theoretical approach can be used to explain many other aspects of social behaviour; the different behaviour of immature males and females, for instance, can be related to

strategies for maximising their fitness as adults.

In contrast, sessions on ecology produced few conceptual syntheses, despite a striking increase in the quality and quantity of research in this field. Detailed accounts of primate communities in Asia, Africa and the Americas were presented, the most notable being that of T. T. Struhsaker (New York Zoological Society) and his associates in the Kibale Forest, Uganda. In all areas, leaf eaters attained higher biomasses than fruit eaters, and these higher than insectivores, but otherwise few general principles emerged. Indeed discussion revealed dissent on aims and methods, let alone conclusions.

We still do not know what factors limit the population of any primate. Availability of food is clearly a plausible candidate; K. Milton's (New York) studies of the howler monkeys of Barro Colorado Island and their habitat suggest that fruit and young leaves of suitable quality may be in short supply at some seasons. Food intake may be limited as much by the need to avoid toxins as to obtain nutrients. Milton showed that many potential foods were rich in phenolic compounds, and J. S. Gartlan and D. B. McKey (University of Wisconsin) likewise demonstrated the presence of toxins in most plant products in the Douala-Edea forest of Cameroon. On the other hand T. Iwamoto (Miyazaki University), in bioenergetic studies of Japanese monkeys in evergreen forest and gelada baboons in Ethiopian montane grassland, obtained figures suggesting that primates used only a tiny proportion of the potential food available. T. H. Clutton-Brock (University of Sussex), and other participants in the concluding discussion, considered that problems of measuring food availability were so intractable, at least in tropical forest, that any attempts were doomed to failure.

S. A. Altmann (University of Chicago) suggested that attention should be focused on species living in relatively simple habitats, and presented a mathematical model of optimal diet, to be tested on savanna baboons in Kenya. For foraging strategies to be adaptive, he pointed out, animals must eat enough of the various foods available to stay above the minimum for every nutrient and below the maximum for every toxin, at the least possible cost. While the model related these factors with elegance and simplicity, many participants thought it likely to founder on the practical problems of measuring cost, including as it does such diverse elements as energy expenditure, time, and risk of predation. Clearly we must wait some years yet before the value of this and competing approaches becomes clear.

One aspect of research on primate social organisation which seems to be relatively underdeveloped is that of communication, which was the subject of a review lecture by Peter Marler (Rockefeller University). It is still not known for certain whether the dozen or so sounds that make up the communicative repertoire of most monkeys and apes are ever used in the way that words are used to make sentences—one sound retaining the same meaning in different sound combinations—or whether it is always the complete pattern that conveys the message, and not the sum of its parts. It is almost certainly the entire sound combination, but the precise interpretation of primate calls in their natural context can be extremely difficult and Marler took the view that the effects of playing back natural calls should be very illuminating, perhaps especially in the laboratory in combination with single-unit recordings from the animals' cortex. (Neurophysiologists, who have had more experience with the problems of interpreting the calls of single units in their natural surroundings, might take issue with him on the latter point.)

What apes have certainly not done is to develop a language in the sense that man has, and in spite of the research resources poured into the attempt, they have not been conclusively shown to be able to use ours in a strictly linguistic way. Although D. M. Rumbaugh (Yerkes Primate Center, Atlanta) protests the contrary, David Premack (University of Pennsylvania) not only conceded that apes never have grasped human language, but is willing to predict that they never will.

They can, however, fairly readily learn to use abstract symbols to represent objects and actions, and they can use the symbols in strings. What is missing is the evidence that the strings are composed according to syntactic rules.

Tired of trying to satisfy professional linguists that ape use of symbols fulfils the criteria for linguistic competence, some researchers have declared they would be satisfied by a "Turing test": if a man cannot tell whether he is conversing with an ape or another man, then the ape is using language. P. A. M. Seuren, collaborating with G. Ettlinger and others at the Institute of Psychiatry (London University), has, as a linguist himself, suggested a more sophisticated test which hinges on an anomaly of human usage. We say, for instance, "Don't do this and don't do that" or "Don't do this or that", but never the logically equivalent "don't do this and that". That linguistic quirk, according to Seuren, is virtually universal in human languages. The question is, would it crop up spontaneously

in ape usage? The answer will not be available until Ettlinger and his colleagues have succeeded in teaching apes to use "or", which has so far not been done. □

Heavy ions at Caen

from P. E. Hodgson

The European Physical Society Conference on Nuclear Physics with Heavy Ions was held at Caen on September 6–10, 1976.

HEAVY ion physics has developed rapidly over the last few years and the conference provided an opportunity to survey the areas that are already quite well understood, and also to discuss the extensive new data on new phenomena, and the theories that have been suggested to account for them. Caen was chosen for the Conference because a powerful new heavy ion accelerator GANIL is to be built there in the next few years.

Glancing collisions between heavy ions, in which one or two ions are transferred, are useful probes of nuclear structure, and the present state of this work was surveyed by O. Hansen and J. Garrett (University of Copenhagen). Detailed theories of these reactions have been developed, and very complicated theoretical calculations, involving the solution of coupled Schrödinger equations, are needed to analyse the data. K. Low (CEN, Saclay) showed how these calculations account for the details of the reaction mechanisms. Their very complexity does, however, make it difficult to use such reactions for nuclear structure studies. This work also needs the optical potentials that represent the interaction between the ions, and some of the latest methods of calculating these potentials were surveyed by D. Brink (University of Oxford).

Extremely complicated reactions take place when the heavy ions collide more nearly head-on. They can fuse together to form a highly excited compound system which then decays by particle emission (discussed by M. Lefort (Orsay)). If the collision is intermediate between glancing and head-on the two nuclei can orbit round each other and exchange a few nucleons, becoming highly excited in the process, before separating again. Measurements have been made of the energies and masses of the ions after these deep inelastic interactions, and some of the results were considered by J. Galin (Orsay) and by L. G. Moretto (Berkeley). The theories that have been developed to account for these data were summarised in a comprehensive review by W. Nörenberg (University

of Heidelberg). The earlier theories using the classical concept of friction are now being replaced by microscopic transport theories and the spreading of the energy distribution is described by the Fokker–Planck equation. This new and detailed understanding of deep inelastic scattering was one of the more important developments reported to the conference.

When two heavy ions combine after a non-central collision, the compound system has a large angular momentum. This preferential excitation of high spin states is a useful means of studying nuclear structure, and the development of our knowledge in this area was surveyed by Z. Szymanski (University of Warsaw).

A special session was devoted to the many attempts that have been made in the past few months in several laboratories to detect superheavy nuclei, after the announcement that evidence has been found for their natural occurrence in the mineral monazite (see *Nature*, **261**, 627; 1976). Samples of monazite have been bombarded with protons and the spectra of the gamma rays subsequently emitted have been measured. Detailed chemical analyses have been made of samples of the mineral and reactions between heavy ions that might lead to their production have been studied. Monazite has been bombarded with heavy ions, and the recoils examined to see if any of them could have come from superheavy nuclei, but none of this work provided any evidence in support of their existence. This does not yet destroy the evidence offered in the original papers, as there are still several important points that require more detailed examination, but the evidence in favour of superheavy nuclei now looks decidedly weaker than it did a month ago. Work will certainly continue in this exciting field. □



A hundred years ago

REFERRING TO OUR recent correspondence on "Antedated Books," a correspondent calls attention to another evil practice that has of late years crept into the publishing trade, namely, that of publishing books without any date at all. Such books are generally of small literary or scientific value, but circulate among a class who are generally unable to test their value. Of course the purpose of issuing undated books is evident; works half a century old may be palmed off on the unknowing as the genuine product of the current year.

From *Nature*, **15**, November 2, 16; 1876.

review article

Development of early stock rearing in the Near East

S. Bökönyi*

The neolithic "revolution", from hunting and gathering in human communities to animal husbandry and agriculture, seems to have originated in the Near East. A primitive kind of domestication arising from the adoption of the young of wild beasts killed by hunters seems to have preceded the deliberate breeding of domesticated strains.

STUDIES on the domestication, origin and development of domestic animals have advanced particularly rapidly in the period since the Second World War. Among those areas to which archaeozoological research has extended essentially only in the past three decades, the Near East (South-west Asia with the southern part of Turkestan) seems to be the most interesting at the moment. There are several reasons. One is that the neolithic "revolution" or "transformation", whose essence was the switch from food gathering to food production, and whose one basic innovation was animal domestication, started in its most interesting and exciting form there. It is also important that the neolithic development of Europe was connected by numerous threads with the neolithic development of the Near East. From this viewpoint too it is especially important that both of the most important domestic species which determined the character of animal husbandry of the earliest Neolithic originated in the Near East.

Before discussing the problems of animal domestication in the Near East, it is useful to begin with a brief summary of the wild forms, their habitat and area of distribution.

The aurochs, the wild ancestor of cattle, spread over the whole Near East in the early post-Pleistocene period. Its remains occur in almost every neolithic sites of the area. The considerable size of these aurochs suggests that the geographical environment must have been favourable. When the plains and hills dried up and were reduced to steppes, the number of aurochs sharply diminished while small isolated populations developed which gradually died out. Around the time of Christ aurochs could be found only in inaccessible mountainous retreats. The typical habitat of the species was the forest steppe or the light forest. It was more an animal of the plains and hilly regions than the bison, its close relative, which was a good climber and preferred dense forests.

Wild sheep occur in abundance in the mountains of the whole Near East excluding the Levant. Their typical habitat is the mountains, though they also live on broad plateaux, climbing over 4,000 m above sea level in summer, and descending into the valleys in winter time.

The bezoar goat, the wild form of our domestic goats, lives all over the mountains from the Levant, through Anatolia, to the Hindu Kush. Its habitat is the high mountains with steep, rugged cliff facings and ravines.

The wild swine was widespread in the whole Near East, in marshy areas and near lakes and streams, in prehistoric times. It still lives in many regions there. It is an inhabitant of wet environments, preferring dense forest, but also living in the bush or among the bush or among the reeds along waterflows.

The wolf, the wild form of the domestic dog, is to be found all over the Near East and shows an extremely wide habitat tolerance that ranges from forest to desert and from mountains to plains.

The habitat preferences of the wild forms described above are a good key to determining in which environmental type of the Near East domestication took place. Steppe and desert can immediately be excluded, for the wolf was the only species which could live there (the dog had no important role in the prehistoric economy of the region). On the other hand, in mountains and their foothills all five species could be domesticated, and pig domestication could also take place in swampy areas of any geographical region.

The domestication of animals was by no means a sudden development, but was preceded by a man-animal relationship which went back hundreds and thousands of years. One must not forget that the relationship between the hunting man and the hunted animal was not simply the killing of the prey. Tens of thousands of hunter generations accumulated and passed on much information on the behaviour, anatomy, biology, physiology, and so on of wild animals, so that the domestication began on well prepared ground. Domestication was undoubtedly connected with a settled life, in common with other aspects of the neolithic revolution. Nevertheless, a settled life was not an indispensable precondition: domestication can happen (and in fact did happen) without settlements, more often however in extreme environmental conditions (for example, reindeer domestication of Arctic peoples), and in such cases real animal husbandry with a wide variety of domestic species never or rarely developed.

It is impossible to resolve the question of whether settled life made domestication possible or vice versa. Probably both are true: it is hardly conceivable that keeping and taming and subsequently breeding a larger number of captured wild animals could occur without corrals or stalls, necessitating at least temporary settled life. It is as inconceivable to supply food to a larger settled community without domestication and animal keeping or at least herding.

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The primary stimulus for animal domestication was probably the drastic climatic and environmental change which followed the end of the Pleistocene. In the Near East the end of the Pleistocene was followed by a short dry period which ended at about 9000–7000 BC. The treeless, sagebrush (*Artemisia*) steppe developed with a trend toward present conditions. It is quite possible that the dry climate and the steppe-desert environment were the direct cause of the withdrawal of the rich wild mammal fauna to the well forested and well watered mountains. Man tried to follow his wild prey, but with the decrease in the number of wild mammals hunting itself became less and less successful. At the same time, man found wild cereals in the mountains and began first to collect and then to plant them. This activity would certainly have required the settlement of at least one part of the population, although the hunters could have continued their seasonal wanderings. It seems more likely, however, that the scale was reduced to a simple change from a summer camp to a winter one. In consequence, however, the wild fauna around the settlement would have thinned out, and man—either consciously or spontaneously—had to look for new sources of animal protein. He found them in animal domestication.

It is, however, quite clear that demographic pressure must also have played an essential part in the changes in food production. Other causes such as the political pressure of more developed groups, the attraction of the products of already food producing communities, and so on, were only of secondary importance in this respect.

Consequently the main aim of domestication was—failing proper methods of meat conservation—to secure living meat reserves. Earlier authors saw the main aim of domestication as the production of enough sacrificial animals to the different gods. Some of these authors have even divided the early domestic animals into groups indicating whether they were the sacrificial animals of the sun god or the moon goddess. This theory certainly does not bear closer investigation, although it is true that some of the domestic species were used for sacrificial purposes at a rather early date. For example cattle horns were found in the shrine of Çatal Hüyük in the fifth millennium BC¹, and at about the same time dog hams were sacrificed by the mesolithic population of Lepenski vir in the Iron Gate gorge of the Danube².

Domestication did not start in one single centre. As man arrived at a particular stage of cultural development at the same time in many areas, he began to control and domesticate certain animal species. Thus each species was domesticated at about the same time in different places and what is designated the earliest site of domestication of a given species can be a matter of sheer chance, depending on where archaeological research has been most successful. On the other hand domestication did not cease after the acquisition of the first domestic animals. It played an important part in increasing domestic stock during the whole Neolithic, it existed in the course of later prehistoric periods, it occasionally appeared even in classical and medieval times and new species are still being domesticated in certain areas of the world.

For a long time it was almost axiomatic that domestication started with the advent of the Neolithic. In more recent times, however, there has been increasing evidence that its earliest beginnings go as far back as the Mesolithic or even late Palaeolithic. In these earlier periods, however, only isolated attempts occurred, with the taming and keeping of individuals of one single animal species that—in spite of all their successes in the domestication of the species in question—never resulted in real animal husbandry exploitation of several domestic species. All these attempts had a common characteristic: their subject was either the dog or (more rarely) the pig. Such animals, whose food requirements were very similar to those of

man, survived on the remnants of human food and needed no substantial vegetable fodder. It was not by chance that in the time of these attempts at domestication plant cultivation did not yet exist, and the idea of securing such fodder therefore could not arise.

Domestication can be traced back to the final period of the Palaeolithic also in the Near East. Turnbull and Reed found domestic dog remains in the Zarzian (final Pleistocene) layer (about 12000 BC) of the Palegawra Cave in North-east Iraq³. Among them is a lower jaw whose crowded premolars prove the domesticated character of the Palegawra dog, making it the earliest known domestic animal. Otherwise in all probability domesticated dogs were found in Natufian (10000–8000 BC) sites of Palestine⁴.

The next two species of the neolithic domestic fauna were acquired at the beginning of the Neolithic, in the "era of incipient cultivation and domestication" (to use Braidwood's terminology). This era signalled the end of the food collecting phase, when man populated a wide variety of environmental zones and "utilised a far greater variety of resources within any given environmental niche" than before. Flannery calls it a "broad spectrum" collecting pattern⁵. This era lasted from about 9000 BC to about 7000 BC and resulted in the domestication of both small ruminants, the sheep and goat, of our domestic fauna.

The small ruminants were ideal subjects for the first experiments of man in domestication. First of all the capture of their domesticable young was comparatively simple, for the killing of adult individuals (which tried to protect their young) was much less dangerous than the killing of an aurochs cow or a wild sow. Besides this, as small animals of non-predatory character, they could be more easily handled, even after they became adult in captivity. Another important point was that their development was much faster than that of the large species, and man could therefore see the results of his experiments sooner. Finally the unexact food requirements of both sheep and goat could be of most importance in the conditions of primitive animal husbandry.

During this time man attempted the control of other small ruminants too; first of all the different gazelles. Enormous numbers of gazelles lived in the Near East in prehistoric times, and even today great numbers of them are still killed. They often live in large herds, and the possession and rational hunting of such a large herd could easily supply even substantial settlements with enough meat. Probably this was the case with Jericho in Palestine whose 2,000–3,000 early neolithic inhabitants could hardly get enough meat from the very small domestic fauna of the settlement. On the other hand, the large number of gazelle bones found in the site suggest well regulated gazelle hunting or possibly herding. But although herding can verge on the brink of domestication it does not necessarily develop into a real animal husbandry. (This was the case with the gazelles, in which morphological changes which could be ascribed to domestication have never been seen.)

The earliest supposed domestication of sheep was described by Perkins in Zawi Chemi Shanidar, North-east Iraq from 10,870 ± 300 yr BP (ref. 7). The sheep of Shanidar do not show any morphological changes, and their domesticated nature has been inferred from the high ratio (about 50%) of the animals killed while still immature, the sudden increase in the proportion of sheep in the fauna and finally the fact that the region of Shanidar is not considered the natural habitat of wild sheep.

It is appropriate to go over the evidence for domestication at this point because the same problems arise in every case of early domestication. The best evidence for domestication is the appearance of morphological changes (such as a decrease in size, changes in the body propor-

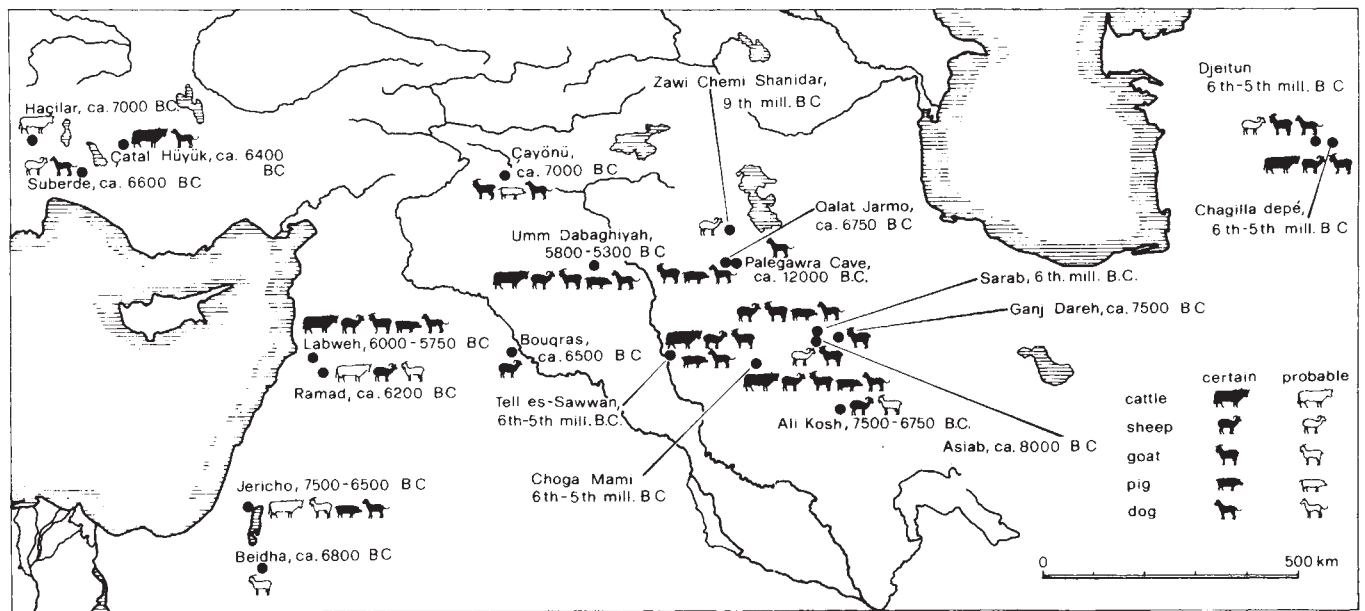


Fig. 1

tions and the form and fine structure of the bones, shortening of the skull, crowded or decreased teeth, hornlessness, changes in the horn form, and so on) in the animals. However, well defined morphological changes do not occur before about 30 generations, according to modern experiments on domestication. The length of a generation is 2–3 yr in small species (dog, sheep, goat, pig) and 5–6 yr in large species (cattle, horse, and so on). Since the earliest domestication of the first domestic species goes back as far as 8,000–14,000 yr, the delay of 60–90 or 150–180 yr in the occurrence of the morphological changes is hardly significant.

But there is indirect evidence of domestication which precedes the appearance of morphological change⁸. This evidence bears on the Near East, because the morphological differences between the wild and domestic forms of the ovicaprids, the most common species there, were not very well known even five years ago. In the late forties and early fifties the high ratio of immature animals alone was accepted as evidence of domestication in the Near East, but it is realised now that it can also be the result of certain hunting methods, and it is accepted as evidence only if it is corroborated by other data.

Further evidence of domestication is the high ratio of adult individuals and particularly of adult males among the remains of the wild form of a domestic species. The explanation is that in specialised hunting connected with domestication man first killed mature individuals, and tried to capture the young ones. At the same time the high ratio of killed males was due to the husbandry of the wild herd where man discovered early that fewer males than females are needed to keep the herd at a given level, and he therefore preferred to kill males if he needed meat.

Animal husbandry increases the occurrence of embryos and newborn animals and also tracks and excrement in settlements. Finally buildings and tools, equipment connected with animal keeping, and representations of domestication scenes of domestic animals, can also be very useful evidence.

Another possible occurrence of early domesticated sheen is in Asiab, West Iran, about 8000 BC (ref. 9). Morphological changes cannot be seen here either, but the very high ratio (80%) of mature animals and the exclusive occurrence of males in the wild sample suggest controlled hunting which might be connected with domestication. On the other hand, the excavation of Ali Kosh, again in

West Iran (7500–6750 BC) yielded remains of undoubtedly domesticated sheep, as well as the skull fragment of a hornless ewe¹⁰. (In prehistoric times there were no hornless individuals among wild sheep; such individuals appear only recently in small, isolated populations.) In the seventh millennium BC domestic sheep appear also in the northern Euphrates area (Bouqras, Ramad) and probably in Anatolia (Suberde) too¹¹.

The earliest domestic goats were found in Asiab, about 8000 BC¹². The occurrence of those domestic goats can be shown by anatomical changes (twisted horns) and the high ratios of mature (82%) and male (95%) individuals among the wild goats killed by the inhabitants. Just a little more recent is the appearance of the domestic goat at Ganj Dareh (7500 BC), in the immediate vicinity of Asiab¹¹. The animal bone sample of this site has not yet been published in detail, but tracks found on the mud bricks of the site point to appearance of domestic goats in the settlement, since wild goats would surely not have come so close to human settlement. As for Ali Kosh, the occurrence of domestic goats in its earliest phase (7500–6750 BC) is uncertain, though probable¹⁰. The domestic goats of Jericho probably also originate from the eighth millennium BC (ref. 13). On the other hand, they can almost certainly be found all over the Near East.

The domestication of cattle and pig, the two latest species in neolithic animal husbandry, occurred during the seventh millennium BC¹⁴. In this era, villages produced most of their food, though hunting and gathering were still important in certain areas. This era began at the end of Prepottery Neolithic and grew into early Pottery Neolithic.

The earliest positively domesticated pigs in the Near East were found in Qalat Jarmo, about 6750 BC (ref. 15). They were fully developed domestic animals with well defined morphological changes. Since there are no traces of a local domestication in the site, one must suppose that they got there from another site where pig domestication took place earlier. The occurrence of domestic pigs is only probable in Çayönü, Anatolia (7000 BC)¹¹ but it is certain in the early levels of Jericho¹³.

The earliest domestic cattle of the Near East are from Çatal Hüyük, Anatolia (6400 BC)¹⁶. Some of the cattle bones of the aceramic level of Hagilar, Anatolia (7000 BC) may also be from domesticated animals (they seem to be smaller than the aurochs bones) but it is still uncertain because of the small sample size¹¹. The occurrence of

domestic cattle in the pre-ceramic phase of Jericho is also not entirely certain¹⁷.

The above descriptions clearly demonstrate that man had completed the domestication of all five species characteristic of neolithic animal husbandry in the Near East by the middle of the seventh millennium BC. On the other hand, it is interesting that the five species did not appear together in any site in the area before the end of the seventh millennium BC, although many different species combinations occurred on many sites. What is particularly curious is that the same five species could be found in several pre-ceramic sites of the Greek islands and mainland Greece around the middle of the seventh millennium BC¹⁸⁻²⁰. This occurred in spite of the fact that out of the five species two (sheep and goat) could not be domesticated on the European mainland because of the absence of wild forms, while two others (dog and pig) were domesticated in the Near East earlier than in South-east Europe. With only one (cattle) does European domestication seem to precede the Near Eastern, although with such a slight time difference (the radiocarbon date of Argissa Magula, Thessaly is 100 years older than that of Çatal Hüyük, Anatolia) that one cannot be sure. Nevertheless, the final word has not yet been spoken on this question because animal bone samples from the pre-ceramic West Anatolia have not yet been published. This region shows the closest genetic connections with the earliest Neolithic of South-east Europe.

As mentioned above, animal husbandry embracing all five domestic species developed in the Near East at the end of the seventh millennium and became widespread during the sixth millennium BC. From this period the faunas of several sites are known, for example Labweh from Lebanon, Umm Dabaghiya, Tell es-Sawwan and Choga Mami from Iraq, Sarab²¹ and Tepé Sabz¹⁰ from Iran, Djeitun and Chagilla depe²² from Turkestan. Their common characteristic is that the caprovines occur as an overwhelming majority in them; the ratios of the other domestic species are rather variable. The ratios of sheep and goat were determined by the geographical environment: in mountainous regions the goat was more common, on plains and among hills the sheep were in the majority. In the settlements of this period also the importance of animal husbandry relative to hunting was quite variable. In general, animal husbandry was more important, but in certain areas hunting remained of decisive importance. In such cases it took a specialised form, such as in Sarab where it was connected with goat domestication, or as in Umm Dabaghiya without local domestication. This latter site is particularly important because, in spite of the fact that its inhabitants kept all five neolithic domestic species, the importance of animal husbandry was minimal, the remains of domestic animals comprising only 10-12% of the animal bone sample. The hunting was specialised for the half-ass (onager) and its bones represent nearly 70% of the sample. This overspecialised hunting did not, however, lead to onager domestication.

The neolithic domestic fauna did not change much even in the further course of the period, and only the sheep gained more ground against the goat. On the other hand, the importance of animal husbandry grew at the expense of hunting which generally declined after the fifth millennium BC. In the third to fourth millennia, the horse and the ass joined a domestic fauna that was gradually completed by new species later, none of which, however, endangered the leading position of the caprovines.

Concerning the early domestic animals of the Near East one may reasonably ask what use they were to neolithic man. As already hinted their original use was almost exclusively for meat. The fact that the first domesticated animals—with the exception of the dog—were derived from species which were originally hunted for their meat has obvious implications. Furthermore, early neolithic man killed most of his domestic animals at an immature age, for example,

caprovines before the first or second winter. The reason was not only that the meat of young animals was more palatable but also that man could provide fodder only for a small, precious breeding stock in the winter fodder shortage. In the advent of domestication and animal husbandry man ate the flesh of every domestic species including the dog whose skulls with opened brain-case (brain was considered a delicacy even in those times) can often be found in the garbage pits of settlements up to the Bronze Age²³. Man did not cease to eat dog meat until the end of the Bronze Age—probably the earliest food prohibition in the world.

The secondary use of hide or fur, tendons, intestines, bones and so on, for tools and clothes was probably established quite early. Only the dog, however, had secondary uses as a live animal, in the roles of hunting companion, watch-dog and herd-dog. In the European Mesolithic it was also used as a sacrificial animal², although this is not yet proven for the Near East. On the other hand, cattle sacrifices in the Near East have very ancient origins. In the shrine of Çatal Hüyük both aurochs and cattle horn-cores were found embedded into clay animal heads²¹, while the bull cult goes back as far as the sixth millennium BC²⁴.

Among real secondary uses of live animals probably milking was the earliest. Animals at a primitive stage of domestication had obviously only enough milk for feeding their young, although they could be milked if their young died or were slaughtered. As prehistoric bone samples witness, death of the young animals was not rare in early animal husbandry, and the young were probably slaughtered so that the suckler animals could be milked (this might also account for the high ratio of animals killed as juveniles in prehistoric settlements). Later, enough milk was produced to give a surplus for human consumption. It is obviously difficult to demonstrate milking from prehistoric material. The earliest representation of milking and milk processing is known from a frieze of the Nin-Hursag temple in Ur (after 2400 BC)²⁵. Interestingly enough the cows are being milked in this scene from the rear, as is usual with goats. From this one may infer that the milking of cows may have emerged after the milking of goats.

Another early secondary use was that of wool of sheep. The body of the wild sheep is covered by coarse kemps among which wool fibres appear only in winter time. These wool fibres are, however, very short and straight. Long, curly wool fibres which do not undergo yearly changes appeared apparently as a result of sudden mutation after domestication. This mutation probably occurred rather early, to judge from a clay sheep statuette found in Sarab (sixth millennium BC)²³ on which the artist has depicted very skilfully the curliness of the wool. Such representations became very common from the fourth millennium BC on, and clay tablets from the turn of the third and second millennium BC often gave sheep shearing lists from estates of Old-Babylonian temples²⁷.

It is still not known how far back the use of draft power of cattle goes. Since there are osteological data in this respect from the Neolithic of South-east Europe, however, one can assume the same too on the Near East. Wheeled vehicles appeared there in the fourth to third millennium BC and they were drawn by cattle. In view of the highly developed state of agriculture in Mesopotamia, cattle-drawn ploughs or some kind of unwheeled vehicle (travois, sledge) could easily have occurred.

The development of early animal husbandry came to an end in the third millennium BC, and at the turn of the third and second millennia, a qualitative change took place. A switchover started from primitive animal keeping to selective conscious animal breeding. This happened on the estates of temples first and first concerned only wool-bearing sheep but later extended slowly to other species as well. As a result of this the size and production of domestic animals grew, their body proportions changed, and new breeds developed which reached remote regions in a short

time and exerted an influence on the domestic animals and animal husbandry as a whole.

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articles

Retrogression rate of the Victoria Falls and the Batoka Gorge

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From an analysis of the archaeological dates for deposits in the Victoria Falls area, we suggest that the rate of erosion of the Victoria Falls was somewhat slower than previously assumed.

ATTENTION was first drawn in the pages of *Nature* in 1905 (ref. 1) to the evidence for the late date and relatively fast rate at which the line of the Victoria Falls, straddling the Zambezi river between Zambia and Rhodesia, reached its present position. In spite of initial challenges, the view was confirmed by subsequent geological and archaeological studies^{2,3} that the gravels now isolated from 110 m to >250 m above the river were laid down by the Zambezi before and during the gradual retrogression of the falls.

The successive positions of the line of the falls are considered to have followed zones of close jointing which formed belts of weakness in the basalt bedrock. These formed steep sided gorges, visible for ~111 km downstream from the Victoria Falls. The archaeological contents of these river-laid deposits above the gorges, or of deposits and events considered to be contemporary, have been used to determine the date by which the falls line had reached different positions³, and thus to calculate the speed of retrogression. There are clearly limits to the reliance that can be given to the geological dating of deposits by their archaeological contents, even where these do not seem to be redeposited. In the absence of much needed new geomorphological studies, however, the archaeology remains our best source of dating evidence. The speeds of retrogression previously calculated^{4,5} gave figures which are equivalent to rates of regression between 3,380 and 5,930 yr km⁻¹.

Such a fast rate of retrogression contrasts with the subjective impression of stability of the falls gained by residents

and visitors. While rock fall is very infrequently observed at the falls line itself, in spite of a mean annual water flow of 3.6×10^9 m³ yr⁻¹, it seems no greater at the line of the western end of the falls, which has been identified⁶ as the beginning of the next line of falls.

The rate of erosion of the gorges was not constant, because of the variable section of the channel in the gorges, and the climatic variation of rainfall in the basin of the upper Zambezi in eastern Angola, western Zambia and northern Botswana. A mean post-Pleistocene rate of 0.29 m yr⁻¹, which could be derived from suggestions⁵ for the date of the erosion of the upper gorges, would, however, seem to require more visible modern erosion.

An empirical test may be made from a generally very accurate topographical illustration by Thomas Baines who visited the falls in 1862. One of his paintings⁷ shows the beginning of the 'new gorge' from the position of the western end of the first gorge. Comparison with the present condition suggests erosion here could not have been more than a few metres in over a century. The view shown represents what today⁸ is a stretch of ~75 m.

Recent reassessments of the dating of Stone Age industries of Southern Africa suggest that the dating of the retrogression of the Victoria Falls based on such industries can now also be reconsidered.

The upper gorges

The date of the erosion of the Fourth Gorge is considered³ to follow the Middle Stone Age occupation of the area. The Kalahari Sand II, actually redeposited Kalahari Sands laid down in a dry period, overlies river-bed gravel to the south of the Fourth Gorge, but not further upstream. This sand, contemporary with what was formerly called the 'Rhodesian Magosian' industry, was laid down before the erosion of the upper three gorges.

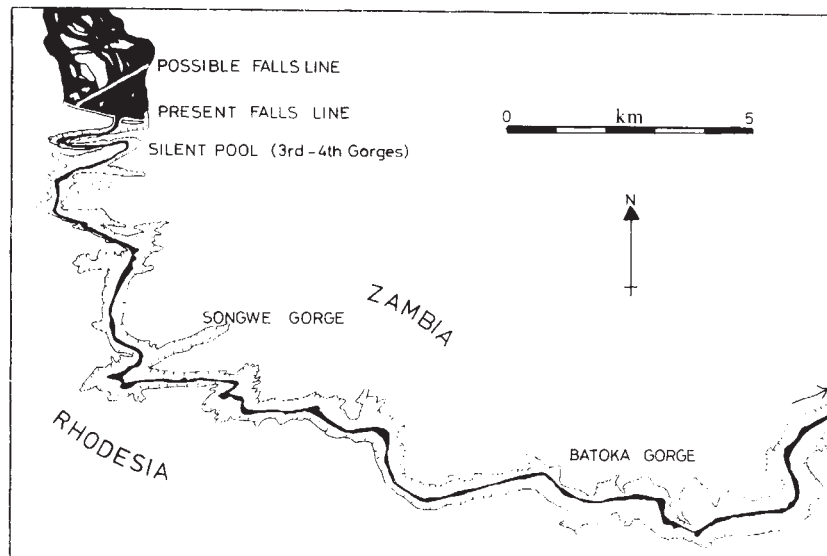


Fig. 1. Map of the falls area.

The distance from the Silent Pool, where the fourth gorge meets the third gorge, to the present day falls is 2.96 km (ref. 8) while the distance from the exit of the second gorge into the falls line to the beginning of the projected new gorge at the western end of the falls is a further 1.17 km, a total of 4.13 km linear retrogression. Estimates of the rate of retrogression have used the north-south erosion following the river, but not the easier lines of erosion which transect this line, and we can therefore use the figure of 2.96 km.

The dates of Magosian and related industries have been reassessed recently. They can no longer be seen as late or terminal Pleistocene cultures making the transition of Middle to Later Stone Age, but rather a long term tradition partly contemporary with other Middle Stone Age industrial traditions⁹. Sites attributed to the Magosian complex and related industries in South Africa have been ¹⁴C dated¹⁰, from >44,500 to >50,250 yr b.p. at Rose Cottage Cave, and 45,900 ± 2,100 yr b.p. at Montagu Cave, but with other dates at the end of the Pleistocene⁹. In Rhodesia dates for Magosian-related material may be later; Pomongwe has a date of 15,800 ± 200 yr b.p. Finds in Zambia which have been grouped with those from the Kalahari Sands II¹¹ include assemblages of 'Proto' Later Stone Age¹² from Leopards Hill dated between 23,800 ± 360 yr b.p. and 21,550 ± 950 yr b.p. Apparently contemporary is the 'Pre-Later Stone Age' industry at Kalembe in eastern Zambia¹³ dated to 24,600 yr b.p. and 24,420 yr b.p.; it is succeeded by a Later Stone Age industry dated to at least 15,330 ± 1,100 yr b.p., while a similar industry at Leopards Hill is dated to 16,715 ± 95 yr b.p.

At Kalambo Falls in northern Zambia a Magosian-related industry named the Polungu¹⁴ has been dated by J. D. Clark at more than 9,550 ± 210 yr b.p., possibly as old as 20,000 yr b.p., or even, by comparison with finds at Zombepata Cave¹⁵ some 20,000 yr earlier than that. At Kalambo Falls, however, an earlier industry of Lupemban type has been dated at 31,660 ± 600 yr b.p., but this date seems younger than should be expected on other grounds; a date over 70,000 yr b.p. for most of the Lupemban may be more probable (K. W. Butzer, personal communication).

Present evidence would suggest the demise of Magosian and related industries in most of Zambia and Rhodesia was considerably earlier than the end Pleistocene date of 12,000 b.p. previously assumed. The most likely time is between 21,000 and 17,000 b.p. with an origin for the complex in the area between 25,000 b.p. and 30,000 b.p. There is as

yet little to support for Zambia or for Rhodesia¹⁰ a date similar to the estimate for South Africa^{10,17} that the Later Stone Age began at ~35,000–20,000 yr b.p. The question of such a date will probably become less conflicting when more detailed study has been carried out on the nature of the industries in the 40,000–12,000 yr b.p. range.

The Kalahari Sands II which predate the erosion of the last 2.96 km of gorge, are therefore dateable to a dry phase within the period defined for the Magosian, at the outside 30,000–17,000 yr b.p., and more probably 30,000–20,000 yr b.p.

Correlated rock falls in Rhodesian caves with archaeological deposits¹⁵ suggest there was a wet period following the end of the 'Stillbay' phase of the Middle Stone Age, and dated at between 25,000 and 21,000 yr b.p. It was followed by a dry phase in which the local variant of the Magosian flourished, a period one can correlate with the Kalahari Sands II of the Victoria Falls, which would thus be dated at later than 25,000 yr b.p. Palaeoclimatic studies at Kalambo Falls¹⁸ suggested close correlation of the temperature curve with that of Europe during late Pleistocene and post-Pleistocene times but the rainfall pattern need not be correlated with temperature change.

Accepting the suggested date limits for the Zambian industries related to the Magosian, and the Rhodesian rock fall, a date for the deposition of the Kalahari Sands II would be between 25,000 and 17,000 yr b.p.

If we place the termination of the Zambian Middle Stone Age at the same time as that now suggested for South Africa, this would allow 20,000 b.p. as a possible date for the start of the erosion of the third gorge, or a mean rate of 6,760 yr km⁻¹ (0.15 m yr⁻¹).

The Zambezi to Songwe Gorge

The dating of the gorges below this point relates to the Younger Gravels and associated earlier deposits attributed to the Upper Pleistocene. The younger gravels themselves contain derived Early Stone Age material and less abraded material from the beginning of the Middle Stone Age, formerly called 'Proto-Stillbay'¹³. The overlying sands and calcretes have a 'Stillbay' industry. Such younger gravels are found at least as far as the top of the gorge above the confluence of the Songwe river with the Zambezi, some 6.75 km below the turn of the fourth gorge into the third, measured along the line of the modern river.

The Younger Gravels were claimed to have been laid

down in a period of high rainfall originally identified with the 'Third Pluvial'¹³.

The river above Songwe Gorge must have eroded back since the period of high rainfall and earlier Middle Stone Age¹⁴; this period had previously been estimated as within the past 50,000–40,000 yr.

The beginning of the Middle Stone Age in Southern Africa has been suggested at ~100,000 yr b.p. (ref. 10) and perhaps over 130,000 yr b.p. as indicated by recent work (K. W. Butzer, personal communication). At Nelson Bay Cave on the Southern Cape coast of South Africa, the Middle Stone Age extended back into the Eem Interglacial probably before 75,000 b.p. (refs 19, 20) and very possibly before 125,000 yr b.p. (K. W. Butzer, personal communication).

The earliest Middle Stone Age radiocarbon date for Zambia is $31,660 \pm 600$ yr b.p. at Kalambo Falls, although the preceding Sangoan was originally dated only six centuries before¹⁴, with an early date of 46,100 yr b.p. Other forms of dating contrast with this picture. This is especially true of the finds of Broken Hill Man at Kabwe in central Zambia. Amino acid racemisation dates for human and associated animal bones at Broken Hill suggest a date of ~110,000 yr bc for Broken Hill Man^{21,22}. Klein²³ argues that the faunal remains probably associated with Broken Hill Man must fall within the Eem Interglacial, or in the Middle Pleistocene (which would imply an earlier date).

The archaeological associations with these dates are unfortunately not completely clear. At the time of Clark's classic study of the Stone Age cultures of the Zambezi valley, however, he attributed³ the finds with Broken Hill Man to 'Proto-Stillbay', and thus a parallel is suggested—whatever terminology be accepted today—between the Broken Hill finds and those of the Younger Gravels. Subsequent reassessment, however, suggests²² the associations may be Sangoan.

The 'Proto-Stillbay' industry was seen as derivative from Sangoan in the Victoria Falls area. It seems, however, that Sangoan industries in southern Africa as a whole overlapped with the earliest Middle Stone Age⁹. The Sangoan at Kalambo Falls was dated by radiocarbon between 46,100 and >32,600 yr b.p., and the preceding Acheulian between >35,000 and $61,700 \pm 1,300$ yr b.p. but these dates now seem much too late. A new minimum date from amino-acid racemisation of >110,000 b.p. (ref. 24) for the Acheulian of the site suggests all the earlier dates for Zambia need to be held in some uncertainty, and probably accepted as *termini ante quos*. The early Middle Stone Age industry of the Younger Gravels at Victoria Falls, which derives from a Sangoan industry, cannot be dated later than 40,000 yr b.p. while in the light of the new dating evidence, a date of <130,000 yr b.p. seems acceptable.

Correlation with the Broken Hill bones and artefacts would suggest a date at ~110,000 yr b.p. for the artefacts of the Younger Gravels. The geological chronology from the southern African coast¹⁹ suggests that the main wet period within the range of the early Middle Stone Age in that area was in the Eem Interglacial before 75,000 yr b.p. Correlations of coastal and inland rainfall patterns are not necessarily reliable, and it may therefore be more useful to rely on the cultural data. Since the erosion above the Songwe Gorge is subsequent to the earlier Middle Stone Age settlement, the falls may have taken up to 90,000 yr to cut back the 6.75 km from the Songwe river to the third gorge, a maximum rate of $13,330 \text{ yr km}^{-1}$ or an average of $11,280 \text{ yr km}^{-1}$ for the past 110,000 yr.

If the retrogression of the stretch from Songwe Gorge were taken to follow the end of the Interglacial, at 75,000 yr b.p., the rate from Songwe to the fourth gorge would be $8,150 \text{ yr km}^{-1}$; an average for the whole distance of $7,725 \text{ yr km}^{-1}$. These alternatives correspond to an average rate of 0.09 or 0.13 m yr^{-1} .

The lower gorges

The area of the gorges below Songwe Gorge has poor access and has not been studied in any detail. The gorges extend ~101 km measured along the river, from the Songwe river to their limit above the confluence with the Matetsi, and the dating of the erosion of this stretch of river is hard to determine. Clark³ originally considered that the gorge, perhaps to 6 km above Chimamba Rapids (a distance that can be calculated at 31 km along the river) was eroded in the Upper Pleistocene, while the lower gorges (70 km) were eroded in the Middle Pleistocene; but a Lower Pleistocene date for the lowest part of the gorges was subsequently suggested⁴. There seems little direct geological evidence for this, and no archaeological data is yet available for the area, although further fieldwork would be useful.

The Middle Pleistocene is now dated²⁵ from 700,000 to 130,000 yr b.p. If we adopt the alternative rates calculated for the retrogression from Songwe Gorge to the modern falls line in the last 75,000 or 110,000 yr, the erosion of the remaining 31 km of the stretch above Chimamba Rapids would have taken 240,000–350,000 years, since 315,000–460,000 yr b.p., well within the Middle Pleistocene.

The presence of Older Gravels of possibly early Middle Pleistocene date near the Matetsi confluence at the lower end of the whole gorge suggests a Lower Pleistocene date for the beginning of the falls retrogression. It seems likely that the erosion of the gorge above the Chimamba Rapids was much more recent than the lower stretch of 70 km (ref. 3), and the geomorphology of the slope and the available evidence on river-laid deposits for this stretch suggests a possible chronological break between the erosion of the lower 70 km and the upper stretch. It is tempting to suggest that this break may have been in the early Middle Pleistocene. The lower stretch from the Matetsi confluence above the Chimamba Rapids, which has geomorphological unity, could all have been eroded within the Lower Pleistocene, over a period (calculated from the rates for the upper stretch) between 540,000 and 790,000 yr since at least $1.25 \times 10^6 \text{ yr b.p.}$, an average of 0.9 m yr^{-1} .

In the absence of well dated contemporary sequences in the region, archaeological dating of the geological events can only be tentative and more detailed geomorphological studies, especially on the lower gorges of the Zambezi, would do much to clarify the situation.

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Transformation by murine and feline sarcoma viruses specifically blocks binding of epidermal growth factor to cells

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Normal cells in culture have membrane receptors for epidermal growth factor (EGF); EGF stimulates cells to divide by binding to these receptors. Cells transformed by murine and feline sarcoma viruses rapidly lose the ability to bind EGF, whereas cells transformed by the DNA tumour viruses, polyoma and SV40, or infected with non-transforming RNA tumour viruses have normal levels of functional EGF receptors. The results suggest that a product of the sarcoma virus genome specifically changes cell EGF receptors; the sarcoma gene product may, then, be functionally related to EGF.

RNA-containing mammalian and avian sarcoma viruses (type C viruses) can produce tumours in a wide variety of susceptible animal hosts and can transform cells from a number of different species in tissue culture^{1,2}. Genetic analyses using temperature-sensitive mutants³ have distinguished specific viral genes (sarcoma genes or oncogenes^{4,5}) essential for transformation. The genomes of the sarcoma viruses contain specific RNA sequences⁶⁻⁸ which are not found in transformation-defective type C viruses. The sarcoma-specific information could code for a protein with a maximum molecular weight of 30,000–40,000. The sarcoma gene product, however, has not been identified; it is presumably synthesised in small quantities and is biologically highly potent. Sequences related to the sarcoma-specific genes have, however, been found as normal constituents of the cellular DNA of both birds⁹ and mammals^{10,11}. From these considerations, we would suggest that the product of the genes should be related to or identical with a normal cellular gene product and that such a protein is (1) a regulator of cell proliferation, (2) of low molecular weight, (3) evolutionarily conserved, (4) produced in only small quantities by sarcoma virus-transformed cells, and (5) synthesised by some normal cells which have not been infected by sarcoma viruses.

One protein that satisfies the criteria for a possible oncogene product is epidermal growth factor (EGF). Mouse epidermal growth factor has 53 amino acids and a molecular weight of 6,045 (ref. 12). It will stimulate the proliferation of various epidermal and mesodermal cells in both whole animals and tissue culture. Injection into neonatal mice accelerates the time of eyelid opening and tooth bud eruption^{12,13}. In organ culture EGF stimulates the growth of epithelial, corneal and mammary gland cells¹⁴⁻¹⁶ and in tissue culture it stimulates cell division of mouse cell lines such as 3T3 and BALB/3T3^{17,18}, mouse and human diploid fibroblasts^{12,19} and human glial cells²⁰. As little as 0.1 ng ml⁻¹ of EGF will stimulate DNA synthesis in human fibroblasts¹². A closely related protein (human EGF) has also been purified

from human urine and shown to have all the biological properties of mouse EGF^{21,22}.

The primary event in growth stimulation induced by EGF (as with other polypeptide hormones²³) is the binding of EGF to specific cell membrane receptors^{24,25}. The factors from both species utilise the same set of membrane receptors in a wide variety of vertebrate cells for their growth stimulatory effects²¹. The genes for both EGF production and its membrane receptors, therefore, seem to have been well conserved in evolution, although the precise normal physiological function of this growth regulatory system is not fully understood.

We now report that in several cell culture systems transformation by mouse and feline sarcoma viruses produces a rapid and profound loss of detectable membrane receptors for EGF. This is not seen in cells transformed by the DNA viruses, SV40 and polyoma, or in cells producing non-transforming leukaemia viruses.

Mouse EGF binding and transformation

Table 1 shows the binding of ¹²⁵I-labelled EGF to various normal and transformed cells in tissue culture. Three different clonal systems of mouse cells were available for study—random bred Swiss 3T3 (ref. 26), BALB/c 3T3 (ref. 27), and NIH Swiss 3T3 (ref. 28). Clonal lines of parental cells were compared with transformed clones produced either by DNA-containing viruses or by RNA-containing viruses. Cells were assayed in their log phase of growth when they were actively dividing, 24–48 h after seeding subconfluent cultures. In each mouse cell system, the Moloney sarcoma virus-transformed cells²⁹ as well as the Kirsten sarcoma virus-transformed cells³⁰ were much less able to bind labelled EGF. One Rous sarcoma virus-transformed BALB/3T3 clone (B77-A31 clone 1) showed only a slightly reduced level of binding compared with normal cells and was quite different in this respect from the MSV-transformed cells.

The 3T3 cell line has been shown to be susceptible to transformation by two DNA tumour viruses, polyoma and SV40 (ref. 31), and, further, doubly transformed clones of 3T3 could be obtained that had integrated both SV40 and polyoma genomes^{32,33}. These cells have all the *in vitro* properties associated with viral transformation and produce tumours in susceptible animal hosts. Whereas MSV-transformed 3T3 clones bind very little labelled EGF the DNA virus-transformed cells show levels of binding not significantly different from the parental cells (Table 1).

When cell lines derived from rat (NRK cells³⁴), mink (Mv1Lu cells³⁵), and cat (FFc60WF³⁶) were studied, cells transformed by the murine sarcoma viruses again showed greatly reduced binding of labelled EGF (Table 1). The normal mink kidney cells, an epithelial line, bound approximately 3–5 times more labelled EGF than did 3T3,

Table 1 Binding of mouse ^{125}I -EGF to normal and virus-transformed cells

Cell line	Transformed by	Designation	Binding ^{125}I (c.p.m. per 10^6 cells)
BALB/3T3	None SV40	A31-CL7	6,700
		SV-A31-CL1	6,900
		SV-A31-CL3	8,700
		SV-A31-CL6	5,900
		M-A31-CL71	310
	MSV-Moloney MSV-Kirsten RSV-B77	K-A31-CL234	140
		B77-A31-CL1	4,300
		A31 (producing R-MuLV)	7,400
		3T3-CL4	4,700
		SV-3T3-CL6	4,300
Swiss 3T3	None	PY-3T3-41	5,200
	SV40	SV-PY-3T3-11	4,600
	Polyoma	M-3T3-CL4	230
	SV40 and polyoma	NIH/3T3	4,800
	MSV-Moloney	NIH/3T3 (producing R-MuLV)	8,300
	None	SV-NIH/3T3-CL1	6,500
	None	SV-NIH/3T3-CL6	7,200
NIH Swiss	SV40	M-NIH/3T3-CL5	200
	MSV-Moloney	K-NIH/3T3-CL65	100
	MSV-Kirsten	NRK-CL2	7,600
	None	M-NRK-CL5-3	230
	MSV-Moloney	K-NRK-CL1	60
	MSV-Kirsten	FSV-NRK-CL3	320
	FSV-Gardner	V-NRK-CL2 (spontaneous rat type C virus producer)	9,800
	None	Mv1Lu (CCL 64)	25,800
	Mink lung	M-MSV-64-CL7	3,200
	None	K-MSV-64-CLJ1	2,300
NRK	MSV-Moloney	FSV-64-CL2	2,800
	MSV-Kirsten	FFc60WF	2,600
	FSV-Gardner	MSV-FFc60	280
	None	FSV-FFc60	230
	None		
Mink lung	None		
	MSV-Moloney		
	MSV-Kirsten		
	FSV-Gardner		
Cat embryo	None		
	MSV-Moloney		
	MSV-Kirsten		
	FSV-Gardner		

The binding assays were performed on subconfluent cell culture monolayers in 60-mm Petri dishes (Falcon 3002). Approximately 1×10^6 cells per dish were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum 24 h before binding ^{125}I -EGF. The cells were washed twice with 2-ml aliquots of binding buffer which consists of DMEM containing 1 mg ml^{-1} bovine serum albumin (BSA) and 50 mM *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES), adjusted to pH 6.8. Binding was performed using 3 ng of ^{125}I -labelled mouse EGF (containing approximately 110,000 c.p.m.) in 1.5 ml of binding buffer. After incubation for 40 min at 37°C , unbound ^{125}I -EGF was removed and the cells washed four times with 2-ml aliquots of cold binding buffer. The cells were solubilised in 0.75 ml of lysing buffer, composed of 0.01 M Tris-HCl, pH 7.4, containing 0.5% sodium dodecyl sulphate and 1 mM EDTA, and the contents transferred to a counting vial. The dishes were washed two more times with 0.5-ml portions of lysing buffer, the contents transferred to the counting vial, and the radioactivity determined using a Beckman 300 series gamma counter. Nonspecific binding was estimated by determining the amount of cell-bound radioactivity in the presence of 10 μg of unlabelled mouse EGF. All data given have been corrected for nonspecific binding. In a typical experiment where 10^5 c.p.m. of ^{125}I -EGF was added, the values for nonspecific binding range from 50–150 c.p.m. above machine background. To test for the possibility that the sarcoma virus-transformed cells rapidly degrade the labelled EGF by proteolytic action, normal and transformed cells were cocultivated in varying ratios and assayed for EGF binding. The binding to normal cells was independent of the number of K-NRK cells present even at a 50-fold excess of transformed cells (2×10^4 normal cells to 1×10^6 transformed cells). When ^{125}I -labelled EGF was first incubated with transformed cells for 60 min and then transferred to normal cells it bound with the same efficiency as freshly-added EGF; no significant degradation of EGF by the transformed cells could be detected.

NRK or cat cells. Although the transformed mink cells bound detectable quantities of labelled EGF, considerably less polypeptide was bound by transformed clones compared with the normal cells (85–95% reduction). Feline sarcoma virus, FeSV (Gardner-Arnstein strain³⁷), transforms the rat, mink and cat cells; non-producer transformed clones have been obtained in all three systems (unpublished).

Cells transformed by FeSV also showed greatly reduced EGF binding, similar to the levels of binding seen with cells transformed by the murine sarcoma viruses. A clone of NRK transformed by avian sarcoma virus (Schmidt-Ruppin strain) showed more nearly normal levels of EGF binding. Reduction in EGF binding to cells derived from several mammalian species is evident after infection by

Table 2 Binding of human ^{125}I -EGF to normal and virus-transformed cells

Cell line	Transformed by	Designation	Binding ^{125}I (c.p.m. per 10^6 cells)
BALB/3T3	None	A31	3,100
	MSV-Moloney	M-A31-CL71	300
	MSV-Kirsten	K-A31-CL234	110
	SV40	SV-A31-CL1	6,800
	SV40	SV-A31-CL6	4,300
	None	NRK-CL2	7,900
NRK	MSV-Moloney	M-NRK-CL537	70
	MSV-Kirsten	K-NRK-CL1	10
	FSV-Gardner	FSV-NRK-CL3	190
	None	Mv1Lu (CCL 64)	24,500
	MSV-Kirsten	K-MSV-64-CLJ1	1,300
Mink lung	FSV-Gardner	FSV-64-CL2	900
	None		

The binding assays were similar to those described in Table 1 except that ^{125}I -labelled human EGF (1.2 ng, 63,100 c.p.m.) was used instead of ^{125}I -labelled mouse EGF. Ten micrograms of unlabelled mouse EGF were used as controls for these experiments. As in Table 1 the nonspecific binding was 50–150 c.p.m. above the counter background.

MSV and FeSV, but not by avian sarcoma viruses or transforming DNA viruses.

The replication of non-transforming type C leukaemia viruses does not affect the ability of cells to bind EGF. Both BALB/3T3 (clone A31) and NIH Swiss 3T3 cells infected with MuLV (Rauscher strain) bind as much ^{125}I -labelled EGF as uninfected cells. Similarly, mink kidney cells infected with feline leukaemia virus³⁷ or with endogenous baboon virus^{38,39} show no reduction in EGF binding (data not shown). Clonal lines of mouse and rat cells such as subclones of A31-7 and NRK clone 2 that can spontaneously produce endogenous type C viruses also bind ^{125}I -EGF as effectively as their parental cell counterparts.

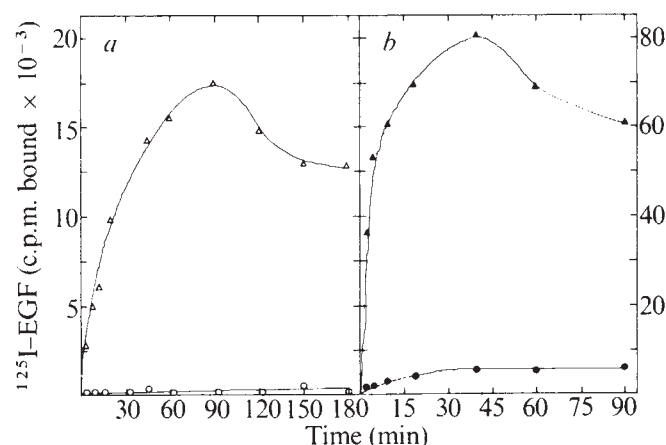


Fig. 1 Time course for the binding of ^{125}I -mouse EGF to normal and transformed cells. The binding buffer consists of Dulbecco's modified Eagle's medium (DMEM) containing 1 mg ml^{-1} of BSA and 50 mM N,N -bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES), pH 6.8. The bindings were carried out on cell monolayers in 60-mm plastic Falcon Petri dishes after washing the plates twice with 1.5 ml of binding buffer. The bindings were then started by the addition of 1.5 ml of binding buffer containing 15 ng of ^{125}I -mouse EGF ($5 \times 10^5\text{ c.p.m.}$) and incubated at 37°C for the stated periods. At the end of the incubations the binding buffer containing the unbound ^{125}I -EGF was removed and the cell monolayers were washed four times with cold binding buffer to remove the excess unbound ^{125}I -EGF. The amount of ^{125}I -EGF bound to the cellular monolayers was measured by lysing the cells and determining the radioactivity present in the lysate. *a*, Binding to normal rat kidney cells: the untransformed parent (Δ), NRK (6.0×10^5 cells per dish) and the Kirsten sarcoma-transformed cell ($\circ$), K-NRK CLI (8.5×10^5 cells per dish). *b*, Mink cells: the normal parent ($\blacktriangle$), MvLu (4.5×10^5 cells per dish) and the Kirsten sarcoma-transformed cell ($\bullet$), K-MSV-64 CLI (5.5×10^5 cells per dish).

Human EGF binding

An epidermal growth factor has been isolated from human urine which shows biological properties similar to those of mouse EGF^{21,22}. Table 2 shows that human EGF also readily detects the difference between MSV-transformed cells, normal cells and SV40-transformed cells. Like mouse EGF, more human EGF binds to the mink lung cells than to BALB/c 3T3 cells and NRK cells. Antisera to mouse EGF cross reacts only weakly with human EGF, and likewise antisera to human EGF cross reacts quite poorly with mouse EGF⁴⁰. Nevertheless, both mouse and human EGF can compete equally well with each other in the binding assays used here with mouse, rat and mink cells, suggesting that the biologically active portion of the molecule involved in binding to receptors has been more conserved during evolution than the major antigenic determinants recognised by antibodies prepared to the mouse and the human EGF.

Time course of EGF binding

Figure 1a shows the extent of binding of labelled mouse EGF to NRK cells, and to NRK cells transformed by murine sarcoma virus, as a function of the time of addition of labelled polypeptide. Maximal binding is achieved for NRK cells at 90 min, after which there is small decline in the EGF bound. This decline is not seen when the binding assays are performed at 23°C rather than 37°C . At no time during the 3-h incubation period at either temperature (in some experiments incubations have been run for as long as 5 h) is there any significant binding of EGF to the sarcoma virus-transformed rat cells. With mink kidney cells (Figure 1b), the time course is slightly different, with peak binding reached earlier, at 40 min, followed by a slight decline. With the transformed mink cells, however, there is a low level of EGF binding, again reaching saturation at 40 min. The transformed mink cells retain some receptors capable of binding EGF even after MSV transformation whereas, with rat and mouse cells, binding is almost entirely abrogated by MSV transformation.

To study the effect of EGF concentration on binding, an experiment was performed using increasing quantities of unlabelled EGF (up to 670 ng of EGF per 10^6 cells) together with the ^{125}I -labelled polypeptide (Fig. 2). No significant binding to the transformed NRK cells was seen at any concentration tested (data not shown). The final extent of binding to mink cells was maximal at approximately 850 pg bound per 10^6 cells for the normal mink cells and 8 to 9 times less for the transformed mink cells. Using this data to obtain a Scatchard plot, recognising that the system is not in complete equilibrium, we calculated that there are 8.4×10^4 receptors per normal mink cell and only 1.0×10^4 receptors per cell in the transformed mink clone. Detectable levels of binding to receptor sites on the transformed and normal cells can be completely inhibited by the addition of unlabelled EGF. Thus, with each of the

Fig. 2 Effect of EGF concentration on binding to normal and transformed mink kidney cells. Three nanograms of ^{125}I -EGF ($210,000\text{ c.p.m.}$) were used in each assay, and unlabelled EGF was added to bring the total EGF to the indicated concentrations. The specific binding was determined after a 40-min incubation at 37°C . $\blacktriangle$, Untransformed MvLu; $\circ$, Kirsten sarcoma virus-transformed progeny K-MSV-64 CLI.

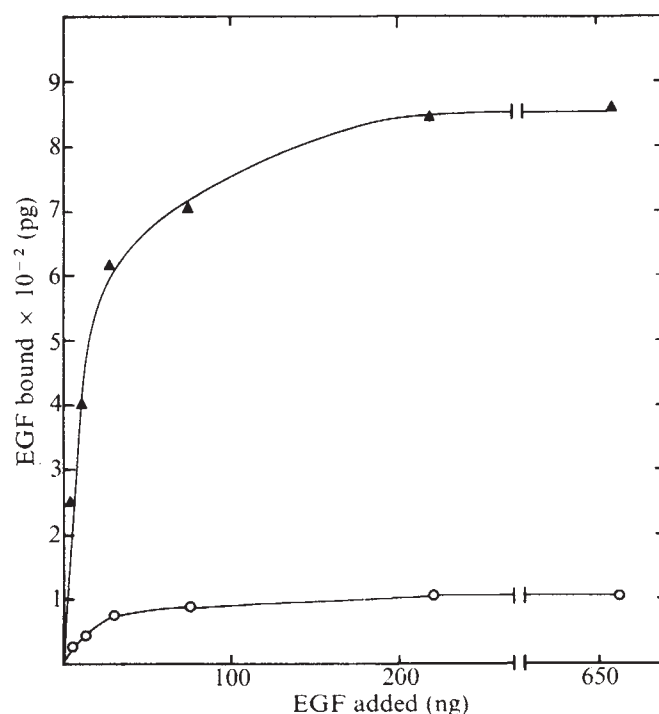


Table 3 Specificity of binding of ^{125}I -EGF to sarcoma virus-transformed cells

Mouse	Cell line	^{125}I (c.p.m. bound per 10^6 cells)		
		EGF	gp70	Con A
	BALB/3T3 untransformed	6,700	2,040	59,500
	MuLV infected	7,400	40	NT
	Sarcoma virus-transformed, virus-producer	170	30	NT
	Sarcoma virus-transformed, non-producer	140	1,910	23,900
	SV40 transformed	5,900	1,820	14,400
Rat	NRK untransformed	7,600	NT	36,900
	Sarcoma virus-transformed, non-producer	60	NT	59,200
Mink	Mink lung untransformed	28,200	NT	64,200
	Sarcoma virus-transformed non-producer	1,500	NT	36,400

The binding assays were performed as in Fig. 1. All proteins were bound to monolayers of log phase cells containing approximately 1×10^6 cells per 60-mm Petri dish. The bindings were performed in binding buffer for 40 min at 37°C . The quantitative differences for the individual ligands used are as follows: mouse EGF, 3 ng of ^{125}I -labelled peptide containing approximately 110,000 c.p.m. and 10 μg of unlabelled material to correct for nonspecific binding; gp70, 6 ng of radiolabelled material containing approximately 68,000 c.p.m. and 10 μg of unlabelled peptide to correct for nonspecific binding; concanavalin A, 10 ng of radiolabelled peptide containing approximately 640,000 c.p.m. and 14.5 mg of alpha methyl-D-mannoside⁴² to correct for nonspecific binding. Of the cell lines tested the SV40-transformed clone (SV-A31-CL6) has consistently given somewhat lower levels of concanavalin A binding compared with both normal and other transformed clones. Whether this is a general property of SV40-transformed clones is not yet clear.

cell lines, it seems that there is one major class of receptors that bind EGF and there is little evidence for cooperative binding or for the existence of a significant fraction of secondary, low-affinity receptors.

Specificity of the cell membrane change

To determine whether the profound alteration in the EGF-binding properties of sarcoma virus-transformed cells is a manifestation of a more generalised membrane alteration, we tested other ligands that bind to cells (Table 3). The major mouse leukaemia virus glycoprotein, gp70, has high-affinity receptors on mouse cell membranes⁴¹. Normal mouse cells in culture can bind gp70, whereas cells that are actively producing type C viruses, either as a result of external infection or as a result of activation of endogenous virogenes, lose the ability to bind the purified viral envelope glycoprotein, because the receptors are occupied by gp70 molecules⁴¹. As shown in Table 3, clone A31 cells have detectable receptors for gp70 whereas cells infected with and producing mouse leukaemia viruses have none. Although sarcoma virus-transformed non-producer cells as well as transformed virus-producing cells do not bind ^{125}I -EGF, only those clones actually producing virus have lost detectable receptors for gp70. SV40-transformation affects neither the binding of gp70 to cell membranes nor the binding of EGF to its specific receptors.

Concanavalin A (con A), binds to cell membranes of

normal and transformed cells⁴². As shown in Table 3, there are only small differences between normal, MSV-transformed, or SV40-transformed cells in the binding of ^{125}I -labelled con A to cell membranes when low concentrations (7 ng ml^{-1}) are used. Insulin receptors have also been reported to show no difference in binding between MSV-transformed and SV40-transformed cells⁴³.

EGF binding after sarcoma virus infection

The loss of detectable EGF binding to MSV- and FeSV-transformed cells suggests that an alteration of EGF receptors is one of the specific effects of infection by some sarcoma viruses at least. However, since the cloned cell lines used in our studies were tested many generations after the initial transformation event, the results could reflect late, secondary effects associated with transformation but not necessarily with the sarcoma virus. To test this possibility, normal mink, rat and cat cells were infected with Kirsten sarcoma or feline sarcoma viruses and EGF binding was assayed as a function of time after infection. An effect on EGF binding was seen as early as 2 d after infection with feline sarcoma virus and 3 d after infection with murine sarcoma virus (Table 4). At this time, the cells were just beginning to show morphological evidence of transformation. By 6 d the effect on EGF receptors was pronounced, and by 2 weeks when most of the cells were clearly transformed, the EGF binding was greatly reduced. Since the

Table 4 Effect of MSV and FSV infection on the binding of EGF to the receptors of various mammalian cells

Cells	Virus	Time (d)	^{125}I -EGF (c.p.m. per 10^6 cells)		
			Control	MSV infected	% of control
MvLu	K-MSV (M28 baboon virus helper)	0	25,800	26,400	102.3
		3	29,400	19,800	67.3
		5	22,800	9,900	43.3
		14	25,500	4,800	18.8
NRK	K-MSV (SSAV virus helper)	0	12,500	14,200	113.6
		3	15,500	7,300	47.1
		6	14,200	4,300	30.3
		14	12,700	700	5.5
Cat embryo (FFc60WF)	GA-FSV (FeLV virus helper)	0	2,400	2,300	95.8
		2	2,800	1,400	50.0
		4	2,400	1,200	50.0
		6	2,500	950	38.0
		14	2,200	320	14.5

At time zero 20% confluent Petri dishes were infected with 1 ml of banded sarcoma virus stock diluted 1:10 with DMEM containing 10% calf serum. The controls were parallel flasks infected in the same manner with helper leukaemia virus free of sarcoma virus. At the indicated time after infection, two dishes each were taken for binding assays as described in the legend to Table 1 using ^{125}I -mouse EGF (3 ng containing 110,000 c.p.m.).

Table 5 Mouse cell virogene expression and oncogenic expression; availability of receptor sites

Cell line	Virus production	gp70 receptor binding	Cell transformation	EGF receptor binding
Mouse embryo diploid	—	+	—	+
BALB/3T3	—	+	—	+
BALB/3T3—MuLV producer	+	—	—	+
BALB/3T3—MSV-transformed, non-producer	—	+	+	—
BALB/3T3—MSV-transformed, MuLV producer	+	—	+	—

mammalian sarcoma viruses are unable to replicate by themselves⁴⁴, sarcoma virus preparations contain mixtures of defective sarcoma viruses and "helper" type C leukaemia viruses. Control cells used in these experiments, therefore, were infected with the helper virus alone (FeLV in the case of the cat cell infection, SSAV⁴⁵ for the rat cell experiment, and the baboon virus M28³⁹ for the mink cell infection). None of the non-transforming viruses could be shown to affect EGF binding. From these experiments we conclude that the alteration in EGF binding to MSV- and FeSV-transformed cells represents an early event following infection that can be demonstrated within two to three cell divisions; in fact, this change may serve as an early index of transformation by murine and feline sarcoma viruses.

EGF receptors and gp70 receptors

BALB/3T3 (clone A31) is not morphologically transformed and is not spontaneously producing endogenous type C viruses⁴⁶. It has 5×10^3 – 6×10^3 leukaemia virus gp70 receptors per cell⁴¹ and 4×10^4 – 5×10^4 EGF receptors per cell (unpublished results). In these properties the clone resembles normal mouse diploid embryo cells (Table 5). After exogenous infection with mouse leukaemia viruses or after activation of endogenous type C viruses the cells no longer bind the viral envelope protein, gp70; they nevertheless continue to bind labelled EGF. Clones of A31 transformed by MSV but not producing virus, on the other hand, retain available receptors for gp70 but no longer bind labelled EGF. Cells that are both transformed by MSV and are producing virus have available receptors for neither gp70 nor for EGF.

EGF as a possible transforming protein

The genes which code for the gp70 receptor as well as those for gp70 itself are endogenous and genetically transmitted in mouse cells. In fact, gp70 has been shown to be a differentiation antigen found on the surface of normal mouse thymocytes^{47–49} and certain other cell types⁵⁰ which are not actively producing type C viruses. Similarly, both the genes for the EGF receptors and for EGF itself must be transmitted as part of the mouse genome. Our results suggest that an alteration in the EGF receptors is a significant early event associated with transformation by murine and feline sarcoma viruses. The decreased levels of EGF binding found in MSV-transformed cells might reflect (1) an indirect effect of the sarcoma gene product on the synthesis, degradation, or location of functional EGF receptors or (2) the production of endogenous EGF or a related substance that binds to the EGF receptors.

One possible model consistent with the data is that the sarcoma genes code for an EGF-related substance and, thus, inhibit binding by added hormone. Sarcoma viruses have arisen by recombination between "helper" type C viruses and sarcoma-specific cellular sequences^{6,8}. Thus, transformation resulting from expression of "sar" genes need not be a consequence of sarcoma virus infection, but alternatively, may result from the expression of endogenous "sar" genes (oncogenes). If the sarcoma gene expression is causally related to the alterations in EGF receptors, the model would predict that certain "non-viral" transformants would also show decreased levels of EGF binding. We have

so far examined 15 chemically-transformed clones derived from BALB/3T3 and have found one benzopyrene-transformed A31 clone that has greatly reduced EGF binding, similar to that seen with the MSV transformants. In this chemically-transformed line, it may be that an endogenous factor, perhaps EGF itself, is produced which can alter the EGF receptor and thereby provide a continued endogenous stimulus for cell division.

Various other polypeptide growth stimulatory hormones such as fibroblast growth factor (FGF)^{51,52}, nerve growth factor (NGF)⁵³, insulin⁴³ and multiplication stimulation activity (MSA)^{54,56} have been described. These also have some of the properties we might expect of a transforming protein. The specific receptors for each of these other hormones are distinct from those that interact with EGF^{12,56–58} and comparisons of DNA virus- and RNA virus-transformed cells have so far only been made for insulin receptors⁴³ where no differences were seen. The EGF binding data described here lead to the conclusion that SV40 and polyoma, each of which also carries a gene(s) for transforming proteins, exert their action elsewhere, and do not involve the EGF receptor-growth regulatory system.

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Partial denaturation mapping of cloned histone DNA from the sea urchin *Psammechinus miliaris*

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A cloned 5.6-kb DNA repeat unit of Psammechinus miliaris containing all five histone coding sequences of known order and polarity has been shown to fall clearly into a low melting and a high melting half by thermal denaturation. The topologies of the DNA sequences thus defined were determined by partial denaturation mapping in the electron microscope to gain further insight into the distribution of spacers and genes within histone DNA.

THE five histone coding sequences of the sea urchin *Psammechinus miliaris* alternate with spacer DNA within a (nominally) 6-kilobase (kb) DNA unit¹ which is tandemly repeated several hundred times^{2,3}. The coding sequences are on the same DNA strand⁴ and are arranged in the order H4, H2B, H3, H2A and H1 (ref. 1). Transcription proceeds in the H4 to H1 direction⁴. In sea urchins, neither the site(s) of initiation of transcription nor the nature of the primary transcript is known, but there is some evidence that HeLa cells contain a high molecular weight nuclear precursor to cytoplasmic histone RNA (M. Melli, G. Spinelli, H. Wyssling and E. Arnold, in preparation).

There has already been extensive restriction mapping¹ and some sequencing (unpublished results) of the histone DNA repeat units of *Psammechinus miliaris*. An alternative approach for the elucidation of the sequence organisation of this DNA is to examine partially denatured DNA molecules in the electron microscope. This will reveal the gross topologies of DNA sequences and will permit the identification of regions of particular interest within the molecule. We have taken this approach and have used for it a protein-free spreading technique⁵, the first time that it has been applied to partial denaturation mapping in a quantitative way.

GC-rich genes and AT-rich spacers

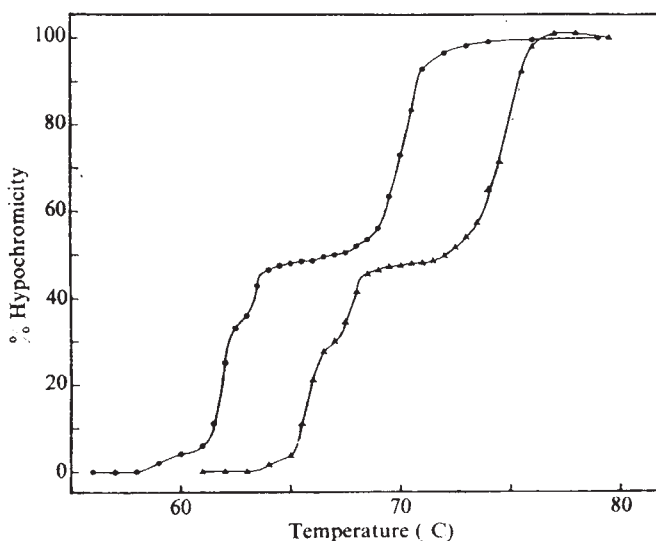
Histone mRNA of sea urchin has been found to be rich in GC (50–56%) by base composition analysis^{6,7}. RNA–DNA hybrids from the histone mRNA subfractions I–III and V of *Psammechinus*, now known to represent the individual mRNAs for H4, H2B, a mixture of H3 and H2A and for H1⁸, respectively, all have the high melting temperature of 75–76 °C in 0.1 × SSC^{2,3}, as would be expected from the high GC content of the sea urchin histone mRNA. By contrast, the intervening spacer DNA is rich in AT. This was first inferred from the banding position (1.704 g ml⁻³; ref. 9) of histone DNA in CsCl and was later substantiated by *T_m* measurements on isolated histone DNA¹. Consequently, histone

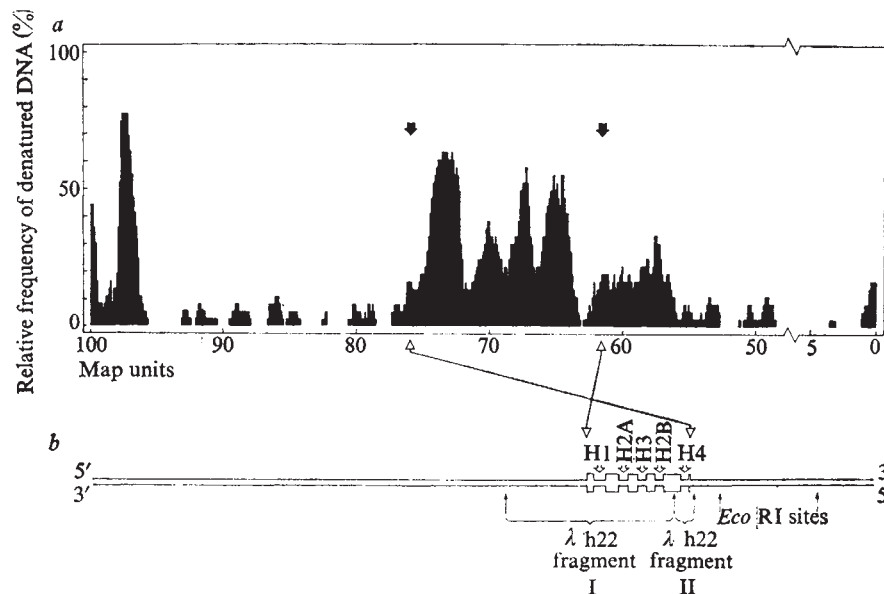
DNA consists of two major types of DNA—GC-rich histone coding and AT-rich spacer DNA sequences.

There are also some definite predictions on the amounts of GC-rich DNA that can be made. The coding sequences for the five histone polypeptides can be calculated to require about 2.13 kb (Table 1). Sea urchin mRNA H4, however, contains additional, untranslated sequences of about 100 nucleotides¹⁰. If stretches of similar size are also found in the other histone messengers, the coding sequences for all five would be of the order of 2.7 kb, or about half the repeat length, now determined to be 5.59 ± 0.35 kb by electron microscopy (see below).

The melting profile of the histone DNA, reclaimed from the recombinant phage λ b538 redB113 imm^{4,34} (Pm histone)-22 (ref. 11) by restriction⁴ is quite simple and has all predicted characteristics (Fig. 1). It falls clearly into early melting and late melting halves. The second major transition has a *T_m* of 74.8 °C (52% GC).

Fig. 1 Melting profile of reclaimed h22 DNA. h22 was isolated from λ h22¹¹ by restriction with *Hind*III and preparative gel electrophoresis as described earlier^{4,20}. The DNA was dialysed exhaustively against 0.1 SSC (▲) or 10 mM NaAc (●), pH 8, and the DNA (10 μ g ml⁻¹) was heated in a Beckman Acta III spectrophotometer. The heating increment was 0.5 °C per min. Bacterial DNAs from *Escherichia coli* and *Micrococcus lysodeikticus* melted at 74 °C and 85 °C, respectively, histone mRNA/DNA hybrids at 75 °C (ref. 3).





Restriction of the recombinant DNA yields λ h22 fragments I and II of 9.1 and 1.1 kb. Others clones, with histone DNA inserted in the opposite way, produce fragments of 4.9 and 5.3 kb (unpublished results of W. Schaffner).

Its thermostability is that anticipated from the base composition of histone DNA and from the T_m of the mRNA-DNA hybrids. The fraction of DNA in this component is consistent with the idea that half the histone DNA is contributed by the coding sequences for the five histone mRNAs, inclusive of untranslated leading and trailing sequences. The other half of DNA melts at 65.6 and 68 °C, respectively, and because of its calculated composition^{12,13} of 34% and 40% GC would not be expected to code for histone mRNA¹. It is taken to represent the AT-rich spacer DNA.

Since the two major melting steps are clearly separated from one another by a pronounced plateau which extends over 4–5 °C, a situation prevails which is ideal for partial denaturation mapping. Conditions can be chosen in which all the AT-rich DNA segments are melted out while leaving the GC-rich regions virtually untouched. The topologies of the DNA segments can then be determined in the electron microscope.

Orientation of the recombinant histone DNA

Partial denaturation mapping in the electron microscope has the disadvantage that it is often impossible to establish the orientation of the gene arrangement from the blister map alone. But 'left' or 'right' of the molecule can be determined if the experiment is performed on uncleaved λ phage DNA into which histone DNA has been inserted in a unique orientation.

The recombinant DNA λ b538 redB113 imm^{4,34} (Pm histone)-22, λ h22 for short, is composed of three elements: the 27-kb left arm, the 10-kb right arm of the receptor phage and the 5.6-kb histone DNA unit intervening between them¹¹. Whenever required, the 5.6-kb histone DNA can be reclaimed from the recombinant DNA by digestion with *Hind* III and fractionation of the three fragments by gel electrophoresis⁴.

The λ -receptor phage contains, in addition to the single *Hind* III site into which the histone DNA was initially inserted¹¹, recognition sequences for a second restriction enzyme, *Eco*RI as indicated in Fig. 2¹⁴. The same enzyme also cleaves histone DNA asymmetrically within the spacer between the H4 and the H2B coding sequence (ref. 1; see Figs 2b and 4b). When the histone DNA recombinant is subjected to *Eco*RI restriction, the products will include two unique DNA fragments which are part histone and part λ DNA. The distances between the *Eco*RI cleavage site within histone DNA and the nearest sites in the λ molecule on either side will clearly depend on the orientation of the histone DNA molecule within the receptor phage. For λ h22, two fragments of 9.1 and 1.1 kb were found by the Southern hybridisation technique (ref. 15; data not shown). For this hybrid phage the orientation given in Fig. 2b applies.

Fig. 2 a, Histogram of 37 partially denatured λ h22 molecules. The frequency of DNA denaturation with map units is given at a resolution of 50 base pairs or 0.12 map units. The areas of the black fields can be taken as a measure of the amount of denatured DNA. In the orientation chosen in Fig. 2a the transcription of the histone genes in the sea urchin would be left to right. The 10-kb 'right' arm of the receptor phage extends from map units 75.8–100%. It includes the typical λ blister¹⁶ at 97.2%. The insertion points for the eukaryotic DNA map at 61.5 and 75.8%, respectively, with a histone DNA intervening between them. On average 37% of the inserted h22 was denatured with blisters appearing at 65.0, 67.5, 70.0 and 73.3%. Even the terminal 80 base pairs near the H4 gene, seen as small fork in Fig. 4, can be detected as blister in intact recombinant DNA in some molecules. **b**, Restriction map of λ h22. The recombinant DNA is shown schematically in the orientation which is standard for λ phage. The large black arrows mark the integration points for h22 DNA which are also the cleavage sites for the *Hind* III restriction enzyme. The arrangement and polarity of the histone-coding sequences within the recombinant has been indicated (see text). There are four recognition sequences for the *Eco*RI restriction enzyme in the λ receptor phage, one in h22.

Denaturation mapping requires heating of the DNA in low ionic strength media, to which formaldehyde is added to prevent snapback of the single-stranded DNA regions. For reclaimed histone DNA in 10 mM NaAc, pH 8, the plateau between low and high melting DNA was found at 60–65 °C (Fig. 1). The effects of formaldehyde cannot be gauged accurately because this agent fixes single-strand DNA regions during thermal breathing of the molecules¹⁶. Using the melting profiles as rough guidelines the conditions appropriate for partial denaturation of the histone DNA molecule had to be determined empirically. In the event, recombinant DNA in 10 mM NaAc, 3.5% formaldehyde, pH 8, was reacted at 61 °C for 10 min, the DNA spread with a protein-free technique (ref. 5; see legend to Fig. 3) and photographed in the electron microscope.

All intact molecules were selected which had the well known, characteristic blister in the distal area of the λ right arm^{16,17} (at map position 97.2%, see Fig. 2a) and the molecules were oriented

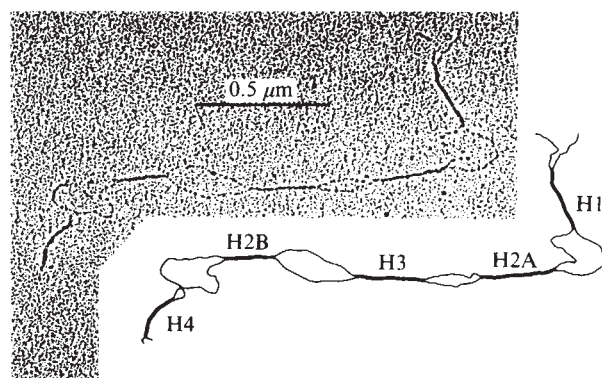


Fig. 3 Partially denatured h22 DNA. For explanation see text. h22 ($10 \mu\text{g ml}^{-1}$) was heated in 10 mM NaAc, 3.5% formaldehyde, pH 8, to 61 °C for 10 min, and cooled rapidly on ice. After 5 min an equal volume of a mixture containing 60% formamide, 180 mM NaAc, pH 8, and $7.2 \times 10^{-3}\%$ benzyltrimethyl-alkylammonium chloride (BAC) was added. 2 μl of the DNA solution was deposited slowly onto the water surface in the centre of a Petri dish (diameter 16 cm) and the spreading of the film monitored by observation of the movement of fine graphite powder placed previously on the water. Carbon-coated 400-mesh copper grids were touched to the surface, the grids washed by floating them on bidistilled water, then stained in uranyl acetate and shadowed with Pt-C. The spreading technique was essentially that of Vollenweider *et al.*⁵. Electron microscopic pictures on Kodak image plates were taken at an instrumental magnification of $\times 8,500$ in a Siemens Elmiskop 101 and the topologies of the DNA segments were determined²⁰ using a map ruler at 1 mm resolution.

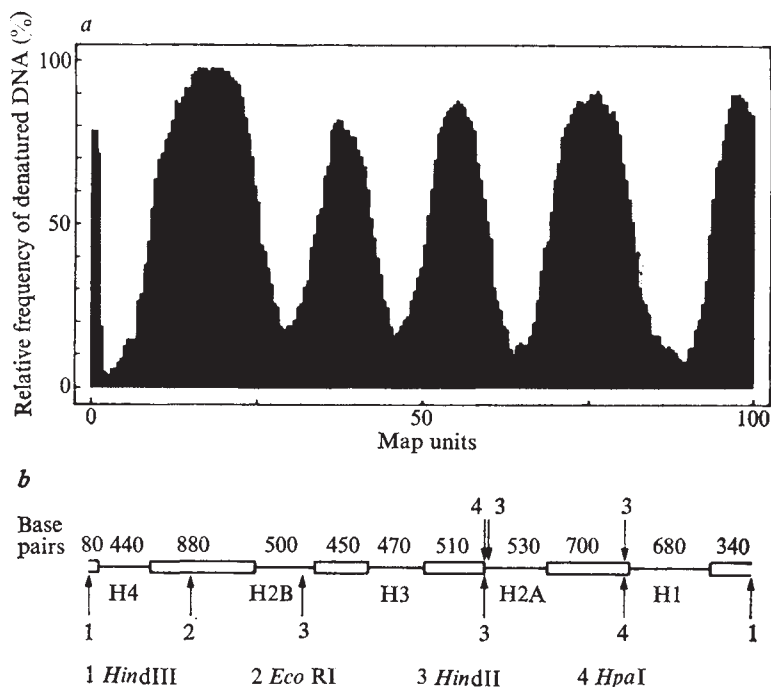


Fig. 4 a, Histogram of partially denatured h22. The diagram depicts the frequency of denatured DNA with map distance calculated from 118 partially denatured h22 molecules. The orientation of the DNA has been chosen on the basis of experiments described in Fig. 2a and b. On average, 53% of the h22 DNA had been denatured as was required on the basis of the melting profile shown in Fig. 1. **b**, Assignments of mRNA coding, spacer and restriction enzyme sequences to the partial denaturation map. The distribution and the average length of DNA in blisters and double-stranded DNA was calculated from the histogram shown in (a). The length of denatured DNA segments were shortened by a factor of 1.1 to correct for the more extensive stretching of single-stranded DNA. The overall length of the molecule was taken at 5.6 kb (see text). The assignment of the coding sequences was made on the basis of published hybridisation data¹. The known cleavage sites of a selection of restriction enzymes^{1,20} have been indicated in the map. 1 = *Hind*III, 2 = *Eco*RI, 3 = *Hpa*I, 4 = *Hind*II. Transcription of the coding sequences is left to right⁴.

with respect to this region. Thirty-seven partially denatured λ h22 molecules were standardised to a hundred map units and the frequency of occurrence of all blisters along the λ DNA molecules were determined. The diagram in Fig. 2a was thus obtained.

Partial denaturation map of histone DNA

First, reclaimed histone DNA was spread for inspection in the electron microscope⁵ and the lengths of the native molecules determined by comparison with added T7 marker molecules. Thirty-five T7 molecules, taken to represent 37.8 kb (ref. 18), averaged out at $12.64 \pm 0.56 \mu\text{m}$. 235 h22 molecules were measured from the same grids. A length of $1.87 \pm 0.12 \mu\text{m}$ or $5.59 \pm 0.35 \text{ kb}$ was found.

With the aim of achieving denaturation of approximately 50% of the DNA molecule, h22 was heated to $61\text{--}62.5^\circ\text{C}$ in 10 mM NaAc, 3.5% formaldehyde, pH 8, and spread for electron microscopy. Partially denatured histone DNA molecules like that shown in Fig. 3 were obtained. The h22 molecule terminates in a small fork at one end, a larger one at the other. Because of this, the denaturation pattern is clearly asymmetrical and so there was no difficulty in orientating the DNA molecules. The denatured and native lengths of 118 molecules in all were measured and their overall lengths were standardised to 100 map units. The frequency of denatured DNA stretches in these molecules is given as a function of map distance at a resolution of 28 base pairs in Fig. 4a.

Anatomy of the cloned histone DNA

The histogram in Fig. 4a shows that the *Psammechinus miliaris* histone DNA h22 consists of AT- and GC-rich DNA stretches intermingled with one another. A small open fork and a small double-stranded stretch are separated, on the left, from a series of three native segments of similar size. Adjacent to these, further to the right, is a middle-sized blister followed by a relatively large unmelted region which terminates, in most cases, in an open fork structure. Since in the sea urchin the 5.6-kb repeat units are joined together without any intervening DNA¹⁹ and are tandemly arranged many hundred times². The two terminal forks can be imagined to be linked in such a way that on denaturation they would form an intact fifth blister on chromosomal DNA.

Except for the joining points between λ and inserted histone DNA, marked by arrows, the blister map of the integrated and the reclaimed isolated h22 DNA shows striking similarities. The black areas in the histogram of Figs 2a and 4a contributed by denatured DNA are clearly different from one another and if numbered 1–4,

from left to right, can be arranged in the order $2 \leq 3 < 4 < 1$ in both diagrams. The polarity of the histone gene cluster in the λ -receptor phage is known (see above). It can be applied to the isolated h22 molecule on the basis of the distinctive blister pattern for both these kinds of molecules. This has been done in Figs 3c and 4b.

For quantitative considerations it is more convenient to discuss the DNA lengths in base pairs rather than in map distances. For this, we have to take account of the fact that denatured DNA⁵ is stretched out by a factor of 1.1 compared with native DNA and hence that the length would be overestimated in an uncorrected linear diagram. The standardised length in base pairs, calculated from a line dissecting the diagram of Fig. 4a at half height, is given in Fig. 4b. The summation of the lengths of all double stranded DNA segments (Table 1) shows that, on average, 53% of each molecule had been denatured.

The partial denaturation map supports the general model for *Psammechinus* histone DNA in which the five genes coding for the five histone proteins alternate with AT-rich spacer DNA. A similar arrangement has been made visible in the electron microscope for histone DNA from another sea urchin by M. Wu, D. Holmes and N. Davidson (California Institute of Technology) using their novel RNA–DNA hybridisation procedure. An intermingling of spacers and genes had previously been suggested by the results of a combined attack on the histone DNA by restriction and molecular RNA–DNA hybridisation¹. In these experiments, it was possible

Table 1 Number of base pairs in histone and histone mRNA coding sequences

Histones	No. of amino acids in protein	Amino acid coding sequence (bp)	mRNA coding sequence (bp)	GC-rich DNA segments (bp)
H4	102 (refs 21 and 22)	306	400 (ref. 8)	440
H2B	125 (ref. 23)	375	ND	500
H3	135 (ref. 24)	405	ND	470
H2A	128 (ref. 24)	384	ND	530
H1	Heterogeneous approx. 220	660*	ND	680
all five		2.62 kb		2.13 kb

Values for H3, H2A and H1 are those for mammals.

*There is some circumstantial evidence^{2,5} that the H1 of the sea urchin may be smaller than that of mammals.

to subdivide the histone DNA repeat unit into five segments of similar size, each of which hybridised to just one histone mRNA but contained a considerably greater amount of DNA than would be required to code for a single histone protein.

In that work, the restriction enzymes *HindIII*, *HindII*, *EcoRI* and *HpaI* were shown to cleave histone DNA between the coding sequences, that is in spacer DNA^{1,20}. Since these cleavage sites have all been mapped by gel electrophoresis of the restriction fragments, their positions can now be found on our partial denaturation map (Fig. 4b). The cleavage sites fall into the blister regions or just within the GC-rich DNA segment near the interphase between melted and unmelted DNA with the exception of one *HindII* site which lies near amino acid 105 of the H2B coding sequence (unpublished results). It has been found that other restriction enzymes cut preferentially in coding sequences, while leaving spacer DNA virtually intact²⁰.

The sequence of 97 nucleotides from the 'left' end of h22 has been determined (unpublished results of H. O. Smith and M. L. Birnstiel). The first 60 of these averaged at a GC content of 34%, while the subsequent DNA stretch contained 50% GC. This demonstrates that the small fork detected in our partial denaturation map near the H4 coding sequence did not arise simply due to breathing of the DNA ends, but reflects the compositional bias of this terminal DNA segment. Here too, there is good accordance between the biochemical and the structural data. Throughout the work we have also noted that the protein-free spreading technique⁵ has provided a reliable means for partial denaturation mapping at high resolution.

Perhaps the most striking correlation that can be drawn is that between the molecular weight of histone proteins and the size of the GC-rich DNA segments delimited on either side by the DNA blisters. The H4 proteins of sea urchins is known to be made up from 102 amino acids^{21,22} and its mRNA to contain some 400 bases^{6,7}. In our map, the DNA assigned to this mRNA is 440 base pairs (Table 1). The largest histone, H1, contains some 220 amino acids²³ and hence some 660 base pairs would be required for its

coding. In keeping with this, the largest double-stranded region is found at the location predicted for the H1 gene. Here, as well as for the middle group of the histone-coding sequences H2A, H2B and H3 there is an excess of base pairs which may code for the leading and trailing sequences of these histone mRNAs. The base sequence arrangement of some of these regions, of spacers and genes will be reported shortly.

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Overlapping genes in bacteriophage ϕ X174

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Bacteriophage ϕ X174 genes D and E are translated from the same DNA sequence but in different reading frames.

BACTERIOPHAGE ϕ X174 contains a single strand of DNA approximately 5,400 nucleotides in length, which has been shown to be of the same sense as the mRNA and to code for nine cistron products. As shown in Table 1, there is an apparent discrepancy between the length of the DNA in the genome and the amount required to code separately for the proteins; significantly more DNA would be needed to code for proteins of the accepted molecular weights than is available. An almost complete sequence of ϕ X DNA has been obtained in this laboratory using the "plus and minus" technique of Sanger and Coulson¹, confirming that the length of the DNA is not more than 5,400 nucleotides (G. M. A., B. G. B., N. L. Brown, A. R. Coulson, J. C. Fiddes, C. A. H., F. Sanger, P. M. Slocombe and M. Smith, in preparation). At the same time, a study of the amino acid sequences of the major ϕ X coded proteins has enabled the boundaries of genes D, J, F, G and H to be located on the restriction map of ϕ X DNA (Fig. 1). Table 1 shows that the molecular weights of the F, G, J and H proteins have been found to be close to correct, while the

D protein molecular weight is somewhat larger than the sodium dodecyl sulphate (SDS) gel estimate. It has not yet been possible to determine directly the boundaries of genes A, B and C in the remaining region between gene H and gene D. The order of genes D to H is in agreement with the genetic map of ϕ X reported by Benbow *et al.*⁴ except that they located gene E between D and J. Here we report the DNA sequence from the promoter preceding gene D through to gene J. We find that gene E amber mutations lie within the DNA sequence which codes for the gene D protein, and interpret this to mean that genes D and E overlap on the DNA sequence. Sequences of gene E amber mutations show that D and E are translated in two reading frames from a common DNA sequence.

It should be emphasised that genes D and E seem perfectly independent by normal genetic criteria. The D protein is produced in large amounts in the infected cell. It is necessary for the production of viral single-stranded DNA, but its precise role in this process is unclear and there is no evidence that it is directly involved in DNA synthesis^{5,6}. Gene E is responsible for lysis of the host cell. All nonsense mutants in gene E characterised so far produce completely functional full size gene D protein. In fact, the protein chemistry of the gene D protein which is reported in this paper has been carried out using material produced by an amber mutant in gene E (*am3*). Similarly, normal gene E function has been observed in the gene D nonsense mutants.

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Table 1 ϕ X174 coding capacity

Gene	Protein molecular weight from SDS gels*	Number of nucleotides From protein molecular weight estimates	From nucleotide sequence	Protein molecular weight from sequence information†
A	62,000	1,701		
(A')	35,000	(960)		
B	19,000–25,000	≥ 522	≥ 1,851	67,400
C	7,000	192		
D	14,500	399	456	16,811‡
E	10,000–17,500	≥ 273	(273)	9,940
J	5,000	138	114	4,097‡
F	48,000	1,317	1,287	46,700
G	19,000	522	525	19,053‡
H	37,000	1,014	981	35,500
Total		≥ 6,078	5,374§	
Length of ϕ X174 DNA		~ 5,370		

*There is considerable variation in reported molecular weight estimates of ϕ X174 coded proteins. The values given are those most frequently recorded, or recently reinvestigated^{1,22,28–33}.

†Protein molecular weights are calculated from the number of nucleotides using the formula:

$$\text{Protein molecular weight} = \text{No. of nucleotides} / (3 \times 0.00915)$$

‡These values are calculated from the amino acid sequences (Freymeyer *et al.*, in preparation; ref. 35 and this paper).

§This figure includes the 160 nucleotides of the intergenic regions so far determined.

Nucleotide sequence analysis of the gene D region

The cleavage sites of ten restriction enzymes of different specificities have been mapped on ϕ X174 RF I (double stranded) DNA. The cleavage sites of these enzymes in the gene D region are shown in Fig. 2.

Using the restriction fragments shown in Fig. 2 as primers with either the (+) or (–) strands of ϕ X DNA as a template, sequences of approximately 100 residues on either side of all the restriction enzyme sites in this region were determined using the plus and minus technique of Sanger and Coulson¹. Figure 3 shows a good example of this method using fragment *Taq* 4 as a primer on ϕ X (–) strand DNA cleaved with *Mbo*II. A long and a short fractionation of the same reactions is shown, from which an almost unambiguous sequence of at least 126 residues can be deduced, starting 14 residues from the *Mbo*II 1/7 site.

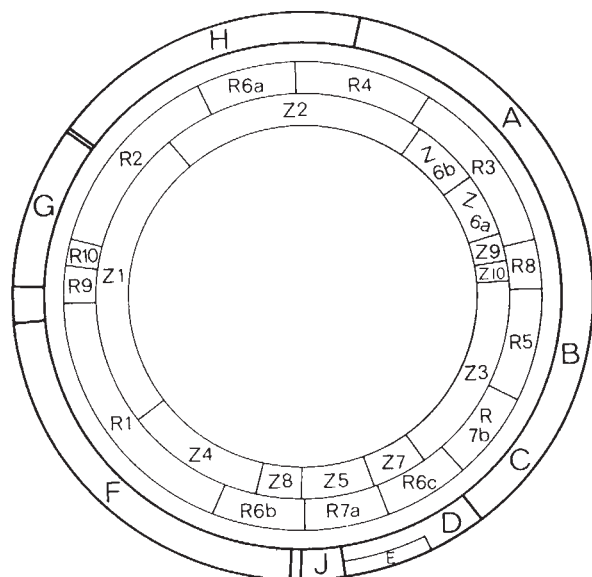
In this way a large number of overlapping sequences from each restriction site and from both strands were obtained which was more than enough to deduce the entire sequence of the gene D region. Although the method is very reliable these overlapping sequences overcame some problems encountered with the plus and minus technique. One disadvantage is that the first 15 residues from the restriction site are not normally seen on the acrylamide gels. This is probably due to the instability of short products on the template which are subsequently degraded by the exonuclease activity of the DNA polymerase present during the restriction enzyme digest of the plus and minus reactions. Another problem encountered is unequal "jump sizes" on the gel between products differing by one residue in length. These occurred in specific regions of the sequence, probably due to local secondary structure at the end of products which were not completely denatured during gel electrophoresis. Electrophoresis at high current (high temperature) gives much improved separation. This problem could also be overcome by priming in the same region from an adjacent restriction site but on the complementary strand. An early difficulty in deducing accurately the number of residues in a run of a particular nucleotide has been overcome by including alongside the plus and minus reactions on the gel a digest of the initial incorporation. This gives a graticule of every possible chain length of the newly synthesised DNA from the restriction enzyme site.

In some cases other techniques such as limited exonuclease digestion of rCTP or rGTP substitution products^{9,10} and depurination of nick-translated restriction enzyme fragments¹¹ have been used to confirm the sequences deduced from the plus and minus system where they were in any doubt. The DNA sequence is shown in Fig. 4.

Amino acid sequence analysis of the D protein

A further confirmation of the DNA sequence was provided by amino acid sequence analysis of the gene D protein. Figure 5 shows the sequences of the protein and of the peptides derived by trypsin, chymotrypsin, thermolysin and cyanogen bromide digestion. The DNA sequence has been used for overlapping several peptides, and amino acids 58, 59, 93 and 123 were not identified in the protein work. The sequence of the first eight amino acids of this protein has been reported by Farber⁶ but it should be noted that our sequence differs by a serine replacing arginine at residue 7. The calculated molecular weight of the protein is 16,811 which is somewhat higher than the estimates from SDS gel electrophoresis of 14,500 daltons

Fig. 1 A map of ϕ X174 DNA. The boundaries of genes D, E, J, F, G and H are shown aligned with the restriction maps of *Hae*III (fragments designated Z) and *Hind*II (fragments designated R)^{2,3}. The precise location of genes A, A', B and C have not yet been determined.



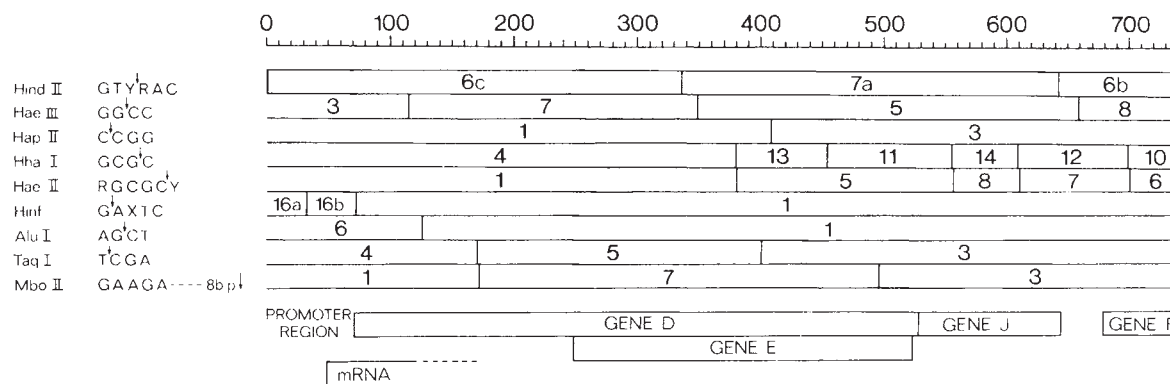


Fig. 2 Restriction map of the gene *D* region. The cleavage sites are shown of *Hind*II, *Hae*III (refs 2 and 3), *Hap*II (ref. 7), *Hha*I, *Hae*II, *Hinf*, *Alu*I (ref. 3), *Taq*I (S. Sato, C. A. H., and J. I. Harris, in preparation), *Mbo*II (N. L. Brown, C. A. H., and M. Smith, in preparation).

(Table 1). In 8 M urea-SDS gels the molecular weight estimate is more accurate at ~16,000 (G. M. A., unpublished).

Location of the promoter and genes *D* and *J*

The amino acid sequence analysis of the gene *D* product unequivocally defines the boundaries of gene *D* on the DNA sequence since termination codons are found in phase before the initiation codon and after the C-terminal amino acid codon. This rules out the possibility of a longer precursor to the gene *D* protein. The gene *D* initiation codon lies within the *Hind*II 6b-*Hae*III 3 region reported by Chen *et al.*¹² to contain an RNA polymerase binding site. Smith and Sinsheimer¹³ have identified the *in vitro* mRNA start from this region as ppp(C) G-A-U-G-C. We find that this sequence occurs 30 residues to the left of the initiation codon of gene *D* (Fig. 4).

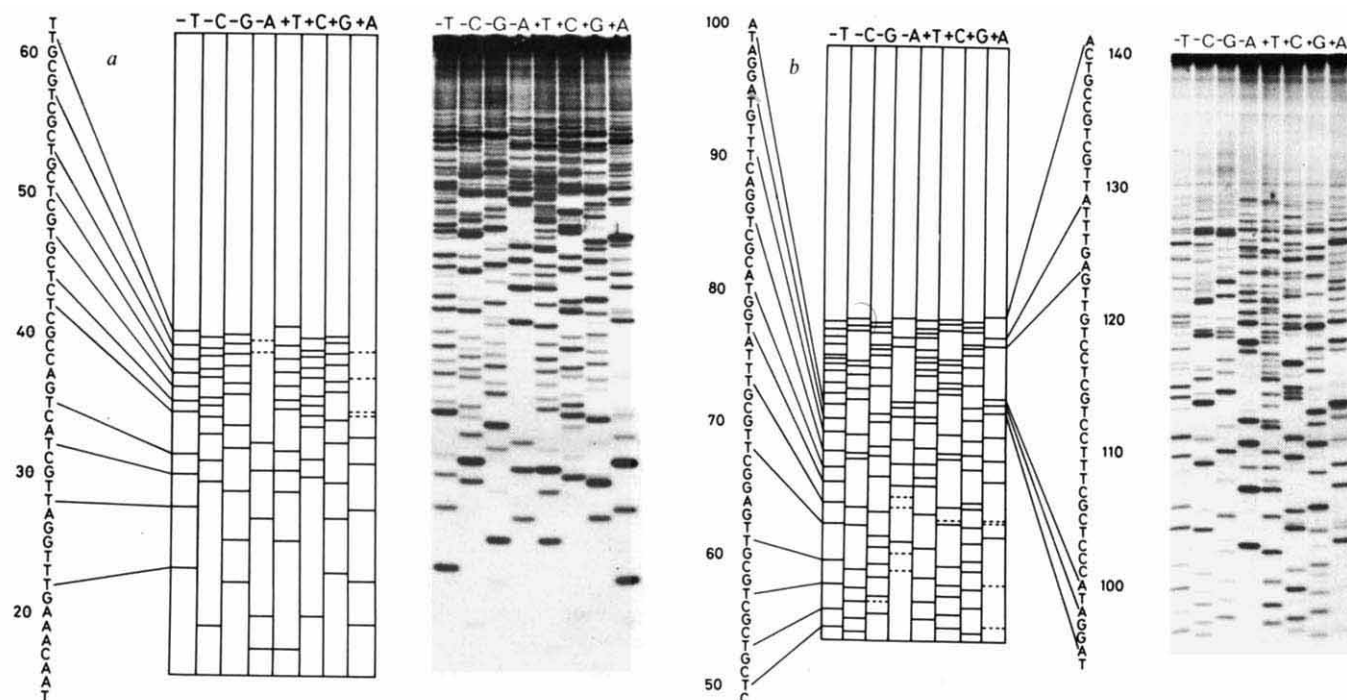
The termination codon of gene *D* overlaps by one nucleotide the initiation codon of gene *J* in the sequence T-A-A-T-G. Overlapping initiation and termination codons have also been

observed in *E. coli* tryptophan mRNA¹⁴. In this case the sequence was shown to be U-G-A-U-G.

Genetic mapping experiments of Benbow *et al.*⁴ suggested the existence of a gene *J* in this region coding for the smallest structural component of the virion. Alignment of the DNA sequence with the amino acid sequence of a small basic protein isolated from the virion (D. Freymeyer, P. R. Shank, T. Vanaman, C. A. H. and M. H. Edgell, in preparation) clearly demonstrates that it is coded by the gene immediately following *D*. We suggest that gene *J* be explicitly defined as the gene coding for this protein since marker rescue experiments show that the mutation (*am6*) which had genetically defined *J* (ref. 4) is located in *Hae*III fragment 7 (ref. 15) which ends 179 nucleotides before the initiation codon of *J* as defined here.

The complete nucleotide sequence of gene *J* has now been determined and has been shown to extend from the end of gene *D* to within approximately 35 nucleotides of the initiation codon of gene *F* (B. G. B., unpublished). This establishes the

Fig. 3 *a*, An autoradiograph of a plus and minus¹ sequencing gel using fragment *Taq*4 as a primer on ϕ X (-) strand template. The products were cleaved with *Mbo*II. A diagram showing the interpretation of the sequence is shown alongside. Electrophoresis was continued until the bromophenol blue marker reached the bottom of the gel. *b*, The same reaction as in *a* but fractionated for a longer time, such that the xylene cyanol marker migrated near to the bottom of the gel.



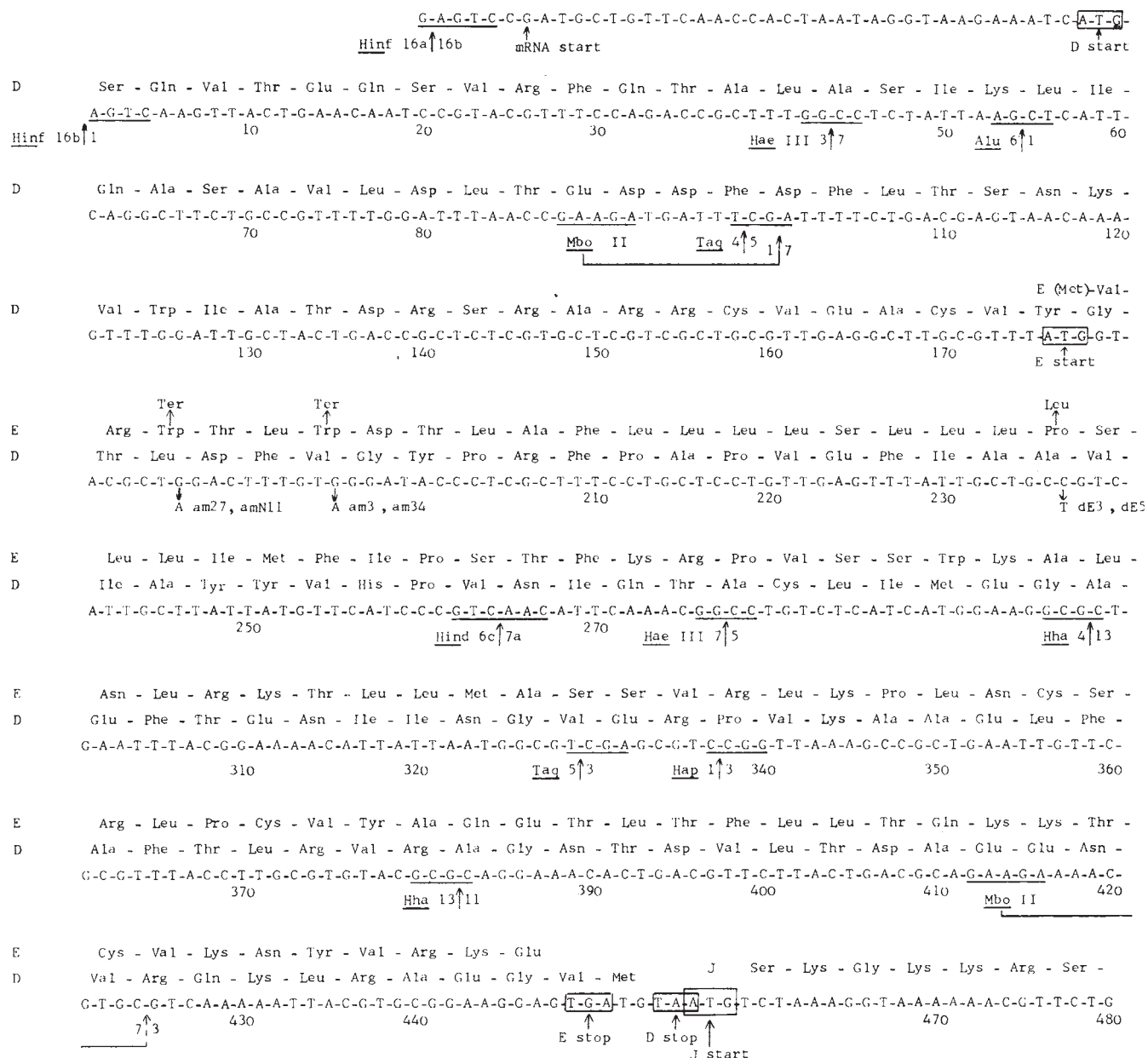


Fig. 4. The nucleotide sequence of the gene *D* region. The amino acid sequences of the *D* protein (see Fig. 5) and the *J* protein are aligned with the DNA sequence. The amino acid sequence of the gene *E* product predicted from the DNA sequence is also shown.

order of genes *D*, *J* and *F* in this region, but gene *E* which mapped genetically immediately following gene *D* (ref. 4) is not accounted for.

Location of gene *E* mutants within gene *D*

It is possible to rescue genetic markers from restriction fragments¹⁶ and thus to establish the location of genetically defined sites on the physical map of ϕ X174. This technique has been applied to locate gene *E*. The double-stranded RF DNA of wild-type ϕ X174 was cut with restriction endonucleases and the resulting fragments were purified by gel electrophoresis. Fragments were denatured and annealed to the viral single-stranded DNA bearing a gene *E* mutation.

Spheroplasts of *E. coli* were transfected with the resulting complexes and the progeny phage were selectively assayed for wild-type plaque-forming particles. Wild-type marker can only be rescued from fragments which span the site of the mutation. Several gene *E* mutants were tested in this way, including *am* nonsense mutants. All were rescued by the wild-type *Hae*III fragment Z7 (Table 2). Weisbeek *et al.*¹⁵ have

also mapped one of these mutants (*am3*) and the mutant (*am6*) (probably a gene *E* mutant) in fragment Z7. Surprisingly, inspection of the nucleotide and amino acid sequence data showed that this fragment is completely contained within gene *D* (Figs 2 and 4). Since gene *E* *am* mutants make a full length *D* protein, these gene *E* nonsense codons cannot be in phase with the translation of gene *D*.

Nucleotide sequence of gene *E* mutants

Sequences within *Hae*III fragment Z7 have been determined for several ϕ X174 strains bearing mutations in gene *E*. Figure 6 compares a gel which displays the sequence synthesised on a viral strand template containing *am3* (gene *E*) with a gel showing the corresponding wild-type sequence. The mutation clearly produces a C → T change in the synthesised minus strand which corresponds to a G → A transition at position 195 in the viral (plus) strand sequence (Fig. 4). Thus the mutation results in the minus strand sequence 5'-C-T-A-3' which corresponds to the amber codon 5'-T-A-G-3' on the viral strand. The phase of this nonsense codon is displaced one nucleotide to the right

	5	10	15	20	25
Sequencer	Ser-Gln-Val-Thr-Glu-Gln-Ser-Val-Arg-Phe-Gln-Thr-Ala-Leu-Ala-Ser-Ile-Lys-Leu-Ile-Gln-Ala-Ser-Ala-Val-				
Tryptic peptides	Ser-Glx-Val-Thr-Glx-Glx-Ser-Val-Arg-Phe Thr-Ala-Leu-Ala-Ser-Ile				
Chymotryptic peptides	Ser-Glx-Val-Thr-Glx-Glx-Ser-Val-Arg-Phe Gln-Thr-Ala-Leu Ala-Ser-Ile-Lys-Leu Ile-Gln-Ala-Ser-Ala-Val-				
Thermolytic peptides	Ser-Glx-Val-Thr-Glx-Glx-Ser Val-Arg Ile-Lys-Leu Ala-Val-				
	30	35	40	45	50
Sequencer	Leu-Asp-Leu-Thr-Glu-Asp-Asp-Phe-Asp-Phe-Leu-Thr-Ser-Asn-Lys-Val-Trp-Ile-Ala-Thr-Asp-Arg-Ser-Arg-Ala-				
Tryptic peptides	Leu Asx-Leu-Thr-Glx-Asx-Asx-Phe(Asx, Phe, Leu) Thr-Ser-Asn-Lys-Val-Trp Ile-Ala-Thr-Asx Ser-Arg Ala-				
Chymotryptic peptides	Leu Asx-Leu-Thr-Glx-Asx-Asx-Phe(Asx, Phe, Leu) Thr-Ser-Asn-Lys-Val-Trp Ile-Ala-Thr-Asx				
Thermolytic peptides	Leu-Asx-Leu-Thr-Glx-Asx-Asx-Phe-Asx-Phe Ile-Ala(Thr)Asx(Arg)Ser-Arg Ala				
	55	60	65	70	75
Sequencer	Arg-Arg-Cys-Val-Glu-Ala-Cys-Val-Tyr-Gly-Thr-Leu-Asp-Phe-Val-Gly-Tyr-Pro-Arg-Phe-Pro-Ala-Pro-Val-Glu-				
Tryptic peptides	Arg Arg Gly-Thr-Leu-Asp-Phe Val-Gly-Tyr-Pro-Arg-Phe-Pro-Ala-Pro-Val-Glu-				
Chymotryptic peptides	Arg-Arg-Cys Val-Glu-Ala-Cys Gly-Thr-Leu-Asp-Phe Val-Gly-Tyr-Pro-Arg-Phe-Pro-Ala-Pro-Val-Glu-				
Thermolytic peptides	Arg-Arg-Cys Val-Glu-Ala-Cys Gly-Thr-Leu-Asp-Phe Val-Gly-Tyr-Pro-Arg-Phe-Pro-Ala-Pro-Val-Glu-				
	80	85	90	95	100
Sequencer	Phe-Ile-Ala-Ala-Val-Ile-Ala-Tyr-Tyr-Val-His-Pro-Val-Asn-Ile-Gln-Thr-Ala-Cys-Leu-Ile-Met-Glu-Gly-Ala-				
Tryptic peptides	Phe Ile-Ala-Ala-Val-Ile-Ala-Tyr Tyr Val Pro-Val-Asx-Ile-Glx-Thr Cys-Leu Ile(Met)Glu-Gly-Ala-				
Chymotryptic peptides	Phe Ile-Ala-Ala-Val-Ile-Ala-Tyr Tyr Val Pro-Val-Asx-Ile-Glx-Thr Cys-Leu Ile(Met)Glu-Gly-Ala-				
Thermolytic peptides	Phe Ile-Ala-Ala-Val-Ile-Ala-Tyr Tyr Val Pro-Val-Asx-Ile-Glx-Thr Cys-Leu Ile(Met)Glu-Gly-Ala-				
CNBr peptides	Phe Ile-Ala-Ala-Val-Ile-Ala-Tyr Tyr Val Pro-Val-Asx-Ile-Glx-Thr Cys-Leu Ile(Met)Glu-Gly-Ala-				
	105	110	115	120	125
Sequencer	Glu-Phe-Thr-Glu-Asn-Ile-Ile-Asn-Gly-Val-Glu-Arg-Pro-Val-Lys-Ala-Ala-Glu-Leu-Phe-Ala-Phe-Thr-Leu-Arg-				
Tryptic peptides	Glu-Phe Thr-Glx-Asx-Ile-Ile-Asx-Gly-Val-Glx-Arg-Pro-Val-Lys-Ala-Ala-Glx-Leu-Phe Ala-Phe				
Chymotryptic peptides	Glu-Phe Thr-Glx-Asx-Ile-Ile-Asx-Gly-Val-Glx-Arg-Pro-Val-Lys-Ala-Ala-Glx-Leu-Phe Ala-Phe				
Thermolytic peptides	Glu-Phe Thr-Glx-Asx-Ile-Ile-Asx-Gly-Val-Glx-Arg-Pro-Val-Lys-Ala Leu-Arg				
CNBr peptides	Glx-Phe-Thr-Glx-Asx-Ile-Ile				
	130	135	140	145	150
Sequencer	Val-Arg-Ala-Gly-Asn-Thr-Asp-Val-Leu-Thr-Asp-Ala-Glu-Glu-Asn-Val-Arg-Gln-Lys-Leu-Arg-Ala-Glu-Gly-Val-Met				
Tryptic peptides	Val-Arg Ala-Gly-Asx-Thr-Asx-Val-Leu-Thr-Asx-Ala-Glx-Glx(Asx)Val-Arg Gln-Lys-Leu-Arg Ala-Glu-Gly-Val-Met				
Chymotryptic peptides	Val-Arg Ala-Gly-Asx-Thr-Asx-Val-Leu-Thr-Asx-Ala-Glx-Glx(Asx)Val-Arg Gln-Lys-Leu-Arg Ala-Glu-Gly-Val-Met				
Thermolytic peptides	Val-Arg-Ala-Gly-Asx-Thr-Asx-Val-Leu-Thr(Asx, Ala, Glx, Glx, Asx) Val-Arg-Gln-Lys				
CNBr peptides					
Carboxypeptidase	Gly-Val-Met				

Fig. 5 Amino acid sequence analysis of the D protein. Sequences of ϕ X174 D protein and peptides derived by tryptic, chymotryptic, thermolytic and CNBr digestion. Solid bars indicate sites of cleavage; the broken bar shows partial cleavage. The residues shown were obtained by automatic (residues 1-17) or manual sequencing. Residues in parentheses were present in the amino acid composition but not identified in the sequencing work. Blanks indicate that the peptide was not sufficiently purified for a reliable amino acid analysis. The protein was isolated from 50 g of frozen ϕ X174-infected *E. coli* C (grown at the Microbiological Research Establishment, Porton Down). Cells were thawed with 50 ml buffer A (20 mM Tris, 10 mM $MgCl_2$, 1 mM mercaptoethanol, 1 mM EDTA pH 8.0), sonicated, and the cell debris centrifuged off, extracted with buffer A and 1 M NaCl, and centrifuged again. The supernatants were dialysed against buffer A at 4 °C, centrifuged again, and the supernatant loaded on a column (3 × 20 cm) of Whatman DE-52 equilibrated with buffer A at 4 °C. After the unadsorbed proteins had been washed through a linear gradient was started (600 ml each A and A + 0.6 M KCl). The D protein eluted about half way through the gradient as monitored by 15% acrylamide gel electrophoresis in 0.1% SDS.

Fractions containing the D protein were combined and 18.7 g $(NH_4)_2SO_4$ added per 100 ml at 4 °C. The resulting precipitate was centrifuged down, resuspended in buffer A, dialysed against buffer A and the suspension centrifuged again. The supernatant contained the D protein sufficiently pure for amino acid sequence analysis. It was dialysed extensively against water and freeze dried.

The amino acid sequencing was by the standard procedures as described previously^{34,35}. The amounts of S-carboxymethylated protein used were 2 mg for N-terminal automatic sequencing in the Beckman sequencer, 5 mg for succinylation and tryptic digestion, 2 mg for chymotryptic digestion, 15 mg for CNBr cleavage, and 5 mg for thermolytic digestion. Purification of the insoluble and the neutral thermolytic peptides was not attempted.

Table 2 Rescue of gene *E* mutants by restriction fragments from gene *D*

Gene <i>E</i> mutant	<i>Hae</i> III fragments			<i>Hha</i> I fragments		
	Z3	Z7	Z5	H8(ab)	H4	H13
<i>am3</i>	1.2×10^3	2×10^4	8×10^2	5×10^2	2×10^4	4×10^2
<i>am27</i>	1×10^2	$> 2 \times 10^4$	2×10^2	—	—	—
<i>am34</i>	6×10^1	$> 2 \times 10^4$	6×10^1	6×10^1	2×10^3	2×10^1
<i>amN11</i>	3×10^1	4×10^3	4×10^1	1×10^1	1.5×10^3	2×10^1
<i>dE3</i>	8×10^1	$> 2 \times 10^3$	6×10^1	4×10^1	1×10^4	4×10^1
<i>dE5</i>	1×10^1	2×10^3	$< 10^1$	$< 10^1$	1.2×10^3	$< 10^1$
S13 <i>amEn15</i>	2×10^2	4×10^3	2×10^2	—	—	—

Restriction fragments of wild-type RF DNA were purified by gel electrophoresis. Fragments were denatured, annealed to viral strand from each of the gene *E* mutants listed, and used to infect spheroplasts of *E. coli* as previously reported¹⁸. Wild-type plaque-forming phage were selectively assayed on *E. coli* C (*su*⁻). The number of wild-type phage per ml following chloroform lysis of the spheroplasts is tabulated, and positive results are underlined. In the experiment shown here only fragments from the gene *D* region were assayed. In another experiment (data not shown) all of the *Hae*III fragments and all of the *Hha*I fragments were assayed for the *am3* site and wild-type markers were only recovered from fragments Z7 and H4. The *am* mutants were assayed on *E. coli* HF4714. The *dE* mutants (see text) were assayed on MacConkey agar (Difco) plates. The phage sample was overlaid in a mixture of 2.5 ml of soft agar, 0.25 ml of 5 mg ml⁻¹ egg white lysozyme, 10 µl of 1 M CaCl₂, and 0.3 ml of an overnight culture of *E. coli* C (*su*⁻). Plates were incubated at 37 °C.

of that used for translation of gene *D*. In this phase a tryptophan codon (T-G-G) is changed to *am* (T-A-G). The mutation does not produce a change in the D product since one valine codon (G-T-G) is changed to another (G-T-A). Similar analysis of an (*am3*) revertant showed that the wild-type sequence was present at position 195.

An independently isolated gene *E* mutant, *am34*, was shown to contain the same altered nucleotide sequence as *am3* (Fig. 4, data not shown). Two other independently isolated gene *E* amber mutants have also been sequenced (*am27* and *amN11*). Both result in G→A transitions on the viral strand at position 186 (Fig. 4, data not shown). This results again in a T-G-G→T-A-G change which produces an amber codon which is in phase with that found in *am3* and *am34*, and displaced one nucleotide to the right of the D translation phase. Again, no coding change is produced in the phase used for D translation (C-T-G→C-T-A, both coding for leucine). The sequence alterations produced by amber mutations at both these sites have been confirmed by pyrimidine tract analysis of the minus strand of *Hae*III fragment Z7. The G→A change reported here for *am* mutations induced by nitrous acid treatment of phage (plus strand) has been predicted by previous studies on the mechanism of nitrous acid mutagenesis^{17,18}.

One observation which seems inconsistent with the results presented here concerns the characterisation of a population of φX174 deletion mutants¹⁹. These deletions were originally interpreted to be located in gene *E* and presumed to be defective only in lysis function. This is inconsistent with our results which predict that any gene *E* deletion would also have a defect in gene *D*. Further analysis of the φX174 deletion population (R. L. Sinsheimer, personal communication) has shown, however, that it must contain deletions from all parts of the genome. We have also investigated this deletion population and obtained evidence that the population does not contain any particles whose only genetic defect is a gene *E* deletion. We observed that all gene *E* *am* mutants tested were able to form plaques efficiently on *su*⁻ hosts when plated on special medium containing bile salts and egg white lysozyme (see legend to Table 2). If simple gene *E* deletions exist it should be possible to clone them on such plates. In fact, about 1–2% of the particles in the deletion population do form plaques on these special plates but not on normal plates on *su*⁻ or *su*⁺ hosts. However, all 20 isolates tested reverted with frequencies reasonable for simple point mutations. Two of these isolates (*dE3* and *dE5*) were mapped by marker rescue on to Z7 and sequenced. Both isolates contain the same missense mutation which produces a predicted Pro → Leu (C-C-G→C-T-G) change in the gene *E* translation phase as defined by sequenced gene *E* amber mutations (position 237, Fig. 4). This would again produce an unchanged D protein since one alanine codon (G-C-C) is changed to another (G-C-T).

Extent of the gene *E* coding region

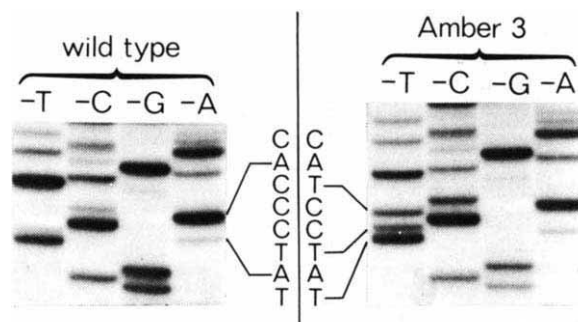
The gene *E* amber mutations are suppressible in *su*⁺ hosts indicating that gene *E* is being translated at those locations. It is therefore possible to determine the boundaries of the gene *E* coding region by looking for termination codons in the same phase as the amber codons. The termination codon at position 449–451 (Fig. 4) defines the 3' terminus of gene *E* protein as it is the first available termination codon in this phase after the amber mutations. Initiation of the gene *E* protein must occur between the termination codon at position 107–109 and the amber mutations. There are two possible initiation codons in this region, a G-T-G at position 146–148 and an A-T-G at position 176–178. Of these the A-T-G is the only initiation codon preceded by a sequence complementary to the 3' sequence of 16S rRNA that has been implicated in the initiation of protein synthesis^{20,21}. Figure 7 shows a comparison of the predicted gene *E* initiation site with other initiation regions found in φX174. Starting at the A-T-G codon at residues 176–178 a polypeptide of 91 amino acids would be synthesised. We have been unable to identify clearly either the E protein or the *am3* fragment *in vivo* or *in vitro*, so amino acid sequence analysis has not been possible. However, the calculated molecular weight of the E protein is in good agreement with the identification of an *E* gene product with a molecular weight of 10,000 by Burgess and Denhardt²², although other estimates have varied (see Table 1).

Evolution of overlapping genes

The mechanism by which the gene *E* product acts to induce lysis of the infected cell is not clearly understood. There is no

Fig. 6 Sequence of the *am3* mutation site. *Hae*III fragment 5 was used as a primer with either wild-type or *am3* φX (+) strand as a template. Only a portion of the autoradiograph of the minus system is shown in both cases with the sequences of the (–) strand deduced. The viral strand sequence is therefore:

wt 5'-G-T-G-G-G-A-T-A-3'
am3 5'-G-T-A-G-G-A-T-A-3'



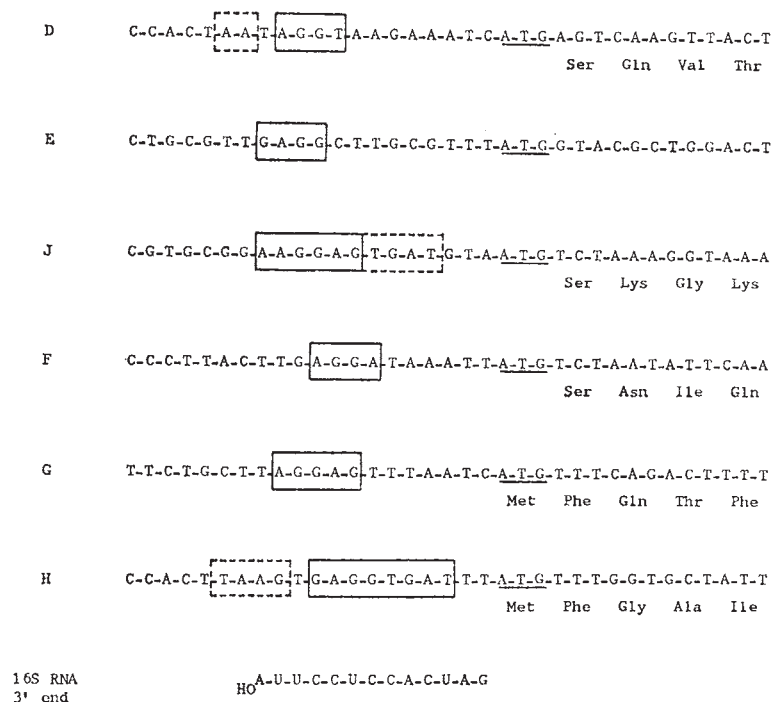


Fig. 7 Comparison of the predicted gene *E* initiation site with other known ϕ X174 initiation sites. The boxed areas indicate sequences complementary to the 3' end of 16S rRNA. Dashed boxes show further complementarity if some nucleotides are looped out or mismatched. Known N-terminal amino acid sequences are also shown.

evidence that it functions as a lysozyme. Bile salts in the presence of egg white lysozyme are able to induce lysis of cells infected with mutants deficient in gene *E* function, although lysozyme alone is not sufficient. This suggests that the gene *E* protein may act on the cell membrane. The predicted amino acid sequence of the *E* protein (Fig. 4) is very hydrophobic in the N-terminal region, in particular in the sequence Leu-Leu-Leu-Leu-Ser-Leu-Leu-Pro-Ser. A large percentage of hydrophobic amino acids including tracts of leucine residues have been observed at the N terminus of precursors to several pancreatic secretory proteins²³ and in the N-terminal region of the light chain IgG precursor²⁴. These N-terminal regions have been proposed to function in the transfer of the proteins across the microsomal membrane. The similarity between these sequences and the N-terminal region of the gene *E* protein is consistent with a membrane-related activity.

An analysis of possible secondary structure in the *E* protein using the rules of Chou and Fasman²⁵ predicts that the N-terminal hydrophobic region could consist of an α -helix terminating before the proline at residue 21. The missense mutations dE3 and dE5 cause a Pro to Leu change at residue 21. This mutation, which causes a loss of lysis function, could affect the secondary structure of the *E* protein by preventing α -helix termination at this point.

It is interesting to speculate on the evolution of overlapping genes *D* and *E*. It is possible that the size of the ϕ X174 genome is limited by packaging or other unknown constraints. If so, the genes required could only be fitted in either by overlapping or by reading from both strands of the DNA. The gene *D* codons contain a high proportion (40%) of T residues in the third position, as is observed in other ϕ X174 genes^{26,27}. This suggests the possibility that gene *D* arose first and that ancestors of ϕ X174 could survive without a specific lysis gene in their natural environment. It is clear that if some mutational event led to initiation of protein synthesis in a phase one nucleotide to the right of the *D* reading phase the resulting codons would have a high proportion of T residues at the second position. Such codons specify hydrophobic amino acids and it seems plausible that a polypeptide could be produced which would interact with the host cell membrane. This could provide a basis for natural selection leading to an efficient lysis gene. It is worth noting that a gene *E* amber mutant in the closely related phage S13 is rescued by fragment Z7 from *wt* ϕ X174 (Table 2).

It seems likely that translation of the gene *D* protein, which is made in large amounts, would obstruct binding of ribosomes at the *E* initiation site. This might be a convenient control if the *E* protein is only required in small amounts. On the other hand, some control may be required to facilitate initiation of the *E* protein, for example, mRNA processing. Initiation of the gene *J* protein poses a similar problem, since the sequence involved in gene *J* ribosome binding is being translated in both *D* and *E* phases.

The fact that genes *D* and *E* overlap alleviates to some extent the problem of fitting the known ϕ X genes into the available DNA (Table 1). However, it is clear that if the molecular weight estimates of the A, B and C proteins are correct, they cannot be separately coded in the region between genes *H* and *D*. Thus, in spite of the severe constraints imposed on the amino acid sequence by translation in two phases, it seems possible that other examples of overlapping genes may be found.

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letters to nature

Determination of the time of the shell ejection in nova outbursts

THE light curve of novae is characterised by a sharp rise in brightness, followed by a decrease in light intensity at an ever slowing rate (see Fig. 1). The optical spectrum of novae near maximum consists of emission and absorption line systems superimposed on a continuum. One of the main features of the evolution of novae spectra is the fading of the continuum relative to the emission lines. In late stages, all novae develop the 'nebular' spectrum, consisting almost exclusively of emission lines with no continuum or with a very weak one (see ref. 1). The analysis of the light curve of a nova can reveal the time of ejection of its main shell. In four galactic novae this time was found to vary between 19 and 40 d before maximum.

Table 1 Two characteristic times of four galactic novae: t_L is the time after maximum light at which the spectrum begins to be dominated by emission lines. T is the time before maximum light of the ejection of the main shell.

Nova	t_L (d)	T (d)
CP Lac	15	19
DI Lac	55	20
Nova Cyg 1975	35	35
Nova Serpentis 1970	95	40

Let $\rho = E_l/E_c$, where E_l and E_c are the energies emitted in the lines and in the continuum. For most novae there is a characteristic time t_L such that for $t > t_L$, $\rho > 1$. From data given in the literature it is very difficult to deduce the value of t_L for novae observed in the past. In four novae, however, whose spectra were recorded at the Wise Observatory in the past 2 yr (Novae: Sag 1974, Per 1974, Scuti 1975 and Cyg 1975) it was found to vary considerably. Inspection by eye of spectrograms of Nova Cyg 1975 leads to an estimate of $t_L \sim 30$ d, after maximum brightness.

By the definition of t_L , the light curve of a nova at $t > t_L$ describes mainly the evolution of the intensity of the emission lines of the nova shell. If the permitted emission lines of novae, in particular those of hydrogen, are produced by ionisation and recombination, the time scale for intensity variations is the recombination time $\tau \approx (N_e \alpha)^{-1}$. Here N_e is the electron density and α is the recombination coefficient on all levels of hydrogen. At an electron temperature $T_e = 10^4$ K, $\alpha \approx 4 \times 10^{-13}$ cm³ s⁻¹ (ref. 2) and for $N_e < 3 \times 10^{17}$ > 1 d. An envelope with a density of 3×10^{11} cm⁻³ ejected at a velocity of 500 km s⁻¹ will reach this density in ~ 50 d. Many galactic novae have a 'transition' period in their light curve, during which rapid oscillations with a time scale of a few hours occur¹. If these oscillations are in the photospheric radiation they must be therefore at times $t < t_L$. There are on the other hand some novae whose light curves are smooth, showing no significant oscillations. It is suggestive to conjecture that in these novae t_L is small. Nova Cyg 1975 is a case in point as the small value of $t_L \sim 30$ d and the light curve in Fig. 1 indicate.

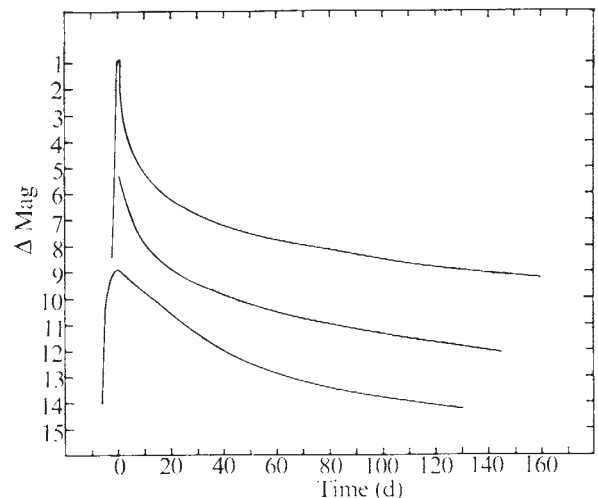


Fig. 1 Smoothed light curves of three novae. The curves of CP Lac (middle) and DI Lac (bottom) are from ref. 1. The curve of Nova Cyg 1975 (top) was constructed by Dr I. Kupo from data published in IAU circulars in 1975 and 1976. The magnitude units are on a relative scale.

The intensity of a recombination line is given by

$$I = \alpha_{\text{eff}} V N_e^2$$

where α_{eff} is the effective recombination coefficient for the emission of the line and V is the emitting volume². The intensity of a forbidden line at a given ionisation and temperature is also proportional to $V N_e^2$. For an expanding ionised hydrogen shell of a given mass, $N_e V = M$ is the mass of the shell in atomic units. Thus

$$I \propto \alpha_{\text{eff}} M N_e$$

For a thin expanding shell of invariant thickness

$$N_e(t) = N_e(0) [r_0 / (r_0 + vt)]^2$$

where $N_e(0)$ and r_0 are the density and the radius of the shell at $t = 0$ and v is the expansion velocity. Therefore

$$\frac{I(t_1)}{I(t)} = \left[\frac{r_0 + vt}{r_0 + vt_1} \right]^2$$

where t_1 is some reference time.

Let the zero of time be the moment of maximum light in the photometric V band, and let T be the time difference between the ejection of the main shell and maximum light. We assume that the acceleration time of the shell is $\ll T$. The ejection time is thus defined more strictly as the time at which most of the main shell acquires its final expansion velocity v . If the assumption is not

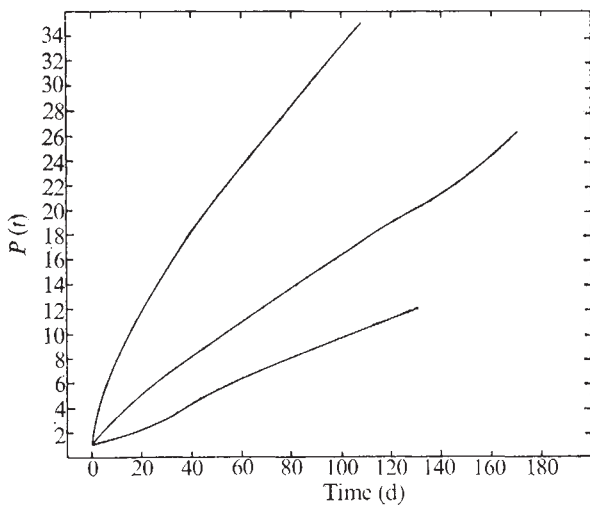


Fig. 2 The observed function $P(t) = [F(0)/F(t)]^{1/2}$ against t for three novae (in same order as in Fig. 1); $F(t)$ is proportional to the observed flux at the time t .

valid, T will be a lower limit to the time difference between ejection and maximum light. We therefore have

$$r_0 \approx \nu T$$

At $t > t_L$ the flux F of the radiation of the nova in the V band is mainly the flux in the emission lines of the shell therefore for $t \geq t_1 \geq t_L$

$$\left[\frac{F(t_1)}{F(t)} \right]^{1/2} = \frac{1+t/T}{1+t_1/T} \quad (1)$$

For all $t > 0$ we define

$$P(t) \equiv [F(0)/F(t)]^{1/2} \quad (2)$$

At $t \geq t_1 \geq t_L$ equations (1) and (2) give

$$P(t) = \alpha t + \beta$$

where

$$\alpha = \frac{[F(0)/F(t_1)]^{1/2}}{T + t_1}; \quad \beta = \frac{[F(0)/F(t_1)]^{1/2}}{1 + t_1/T} \quad (3)$$

A plot of $P(t)$ against t should become a straight line at $t > t_L$. The parameters of the line give the time of the ejection before maximum light

$$T = \beta/\alpha$$

Figure 2 is a plot of $P(t)$ against t for the novae CP Lac, DI Lac and Nova Cyg 1975. These novae have no transition periods in their light curves, as shown in Fig. 1.

The curves in Fig. 2 do have linear sections and the parameters derived from them are summarised in Table 1. The parameters for Nova Serpentis 1970 are obtained from a similar analysis of the light curve of this nova, given by Hutchings³. The value of T for this nova is much less certain because of the small extension of the light curve beyond t_L .

The reality of the theory put forward here and of the results obtained from it is somewhat confirmed by the shape of the curves in Fig. 2 which follows the expected one. In particular it justifies empirically the assumption of invariant thickness of the expanding shell. An homologous expansion of a thin shell, for example, requires a change in the definition of the function $P(t)$ in expression (2) to $[F(0)/F(t)]^{1/3}$. With such a definition, the fit

of $P(t)$ to a linear expression in t is worse than the one obtained here. This is indicated by a smaller correlation coefficient for the fit for the four novae and by the systematic deviation of the distribution of the points from a straight line. Physically this assumption is justified because the velocity dispersion within gaseous shells of novae is probably much smaller than the expansion velocity. At times when the increase in the thickness of the shell is still small relative to the initial thickness, the thickness of the shell is to a first approximation invariant. An additional test can be performed by deriving the value of T directly from the value of α or from the value of β . Using expressions (3) we indeed obtain consistent values of T from both expressions. This is not a trivial result as α and β are two independent observational parameters.

An obvious improvement of the method suggested here is the recording of the intensity of a specific recombination line, for example H_{β} , with narrow band photometry, throughout the nova episode. Data points on such a light curve should be significant for this analysis even at $t < t_L$. They are also unaffected by the radiation in forbidden lines which is much more temperature dependent.

I am grateful to Dr I. Kupo for providing me with the light curve of Nova Cyg 1975.

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Radioactivity in the galactic plane

WE report here what may be the first detection by a nuclear γ -ray transition of a large concentration of interstellar radioactivity. Such a concentration of radioactivity would be expected if heavy nuclei are synthesised explosively in supernovae. We do not have sufficiently conclusive counting statistics to make an incontestable claim, but the phenomenon we have observed in the background of our measurements is tantalisingly near to theoretical expectations.

The observation was made during balloon-altitude measurements of γ -ray energy spectra in the 0.02–12.27 MeV energy band from galactic and extragalactic sources. These measurements were conducted from Rio Cuarto, Argentina, during April 1–2, 1974. The measurements and the actively-collimated scintillation counter we used have been described elsewhere^{1,2}. In each measurement, a given source was observed for many time segments (each averaging 10 min) that we alternated with equal duration, equal zenith-angle measurements of the background. A galactic co-ordinates plot of the relevant background pointing directions is shown in Fig. 1 of ref. 1; the background series starts with segment 1 and ends with background segment 22. The tenth, eleventh and twelfth of these sequential background observations (hereafter labelled B10, B11, and B12) were made in directions towards the plane of the Galaxy—B10 has the lowest average galactic latitude—while the other backgrounds were measured at higher galactic latitudes. B10, B11 and B12 displayed enhanced counting rates relative to the other background segments, and an effort was made to ascertain the cause of the enhancement. This enhancement was most significant statistically at energies < 0.2 MeV, and most significant of all for B10. An energy spectrum was computed for B10 by treating it as resulting from a 'source' and subtracting from B10 a background (B8) measured ~ 30 min earlier at a higher galactic latitude. The 'B10 energy spectrum' is the product of this difference

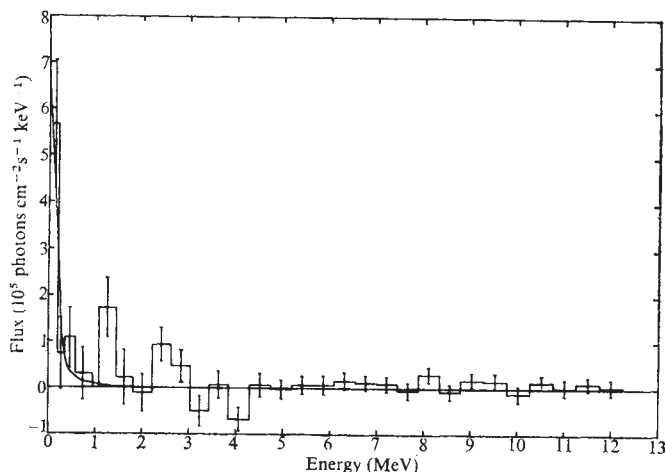


Fig. 1 B10 energy spectrum for April 2, 1974. Data shown are energy-dependent summations of individual pulse-height channels; the F.W.H.M. energy resolution at energies near 1 MeV is 5 individual channels, for example. Bins in the figure are 4.0 F.W.H.M. in energy < 1.0 MeV and 2.0 F.W.H.M. > 1.0 MeV. The best-fit power law (see text) is also shown. All error bars shown are $\pm 1\sigma$ and are purely statistical in character.

multiplied by the appropriate correction factors (Walraven *et al.*³) for atmospheric absorption and instrumental efficiency. Figure 1 shows the B10 energy spectrum with individual pulse-height channels summed (for clarity) as indicated in the caption.

When a power-law fit was attempted to all of the channels in the B10 energy spectrum, a reduced χ^2 value of 0.894 was obtained, for 64 d.f. This fit is $(9.5 \pm 2.6)^{-2.4 \pm 0.1}$ photons $\text{cm}^{-2} \text{s}^{-1} \text{keV}^{-1}$, where the photon energy, E , is expressed in keV. This off-the-galactic-centre continuum has a much harder spectral index than the index of 2.78 found¹ for the galactic centre region continuum. The present continuum spectrum equals, within statistics, a prolongation of the galactic-plane spectrum measured at higher energies on SAS-2 (ref. 4), allowing for our 0.04-sr acceptance angle.

Our curiosity was aroused by the spectral feature that seems to be present (Fig. 1) in the B10 spectrum in a 1.0 F.W.H.M. interval centred near an energy of 1.16 MeV: statistically, it is the only large deviation from the power law. (The spectral lines from excited, stable nuclei that we detected¹ from the central longitudes of the Galaxy are too faint to have been detectable in this brief observation.) There is a flux of 4.2×10^{-3} photons $\text{cm}^{-2} \text{s}^{-1}$ in this interval, which extends from 1.094 to 1.274 MeV. This flux is 2.6σ above that expected from the power law. A hypothesis of a Gaussian superimposed on the B10 power law was fitted to the individual channel data, and a flux amplitude of $(3.4 \pm 1.5) \times 10^{-3}$ photons $\text{cm}^{-2} \text{s}^{-1}$ was obtained, with the hypothetical line at an energy of (1.15 ± 0.07) MeV. This yielded a reduced χ^2 value of 1.195, for 12 degrees of freedom.

Instead of B8 alone, all backgrounds except B10 were combined into a weighted mean, and the weighted mean was then subtracted from B10 to form an energy spectrum. This procedure may not have very great physical significance, because the backgrounds were measured at various zenith angles and at different times. Because the weighted-mean counting rate is slightly higher than that of B8 in this energy interval, this weighted-mean spectral line flux— $(1.9 \pm 1.2) \times 10^{-3}$ photons $\text{cm}^{-2} \text{s}^{-1}$ —is somewhat lower than the line flux given above. The feature clearly lies in a positive excess in B10, rather than a negative departure in either B8 or the weighted-mean backgrounds. Because the statistical confidence is so low, $< 3\sigma$, meaningful comments on possible broadening beyond the instrumental broadening cannot now be made.

B0 seems to have an anomalous energy spectrum. The midpoint of segment B10 occurred when the axis of the detector was pointed towards galactic longitude 345° and the galactic latitude $+5^\circ$. (The estimated error in both coordinates is $\pm 1^\circ$.) In the B10 sky region, the beam of the experiment projects on to the sky as a circle with a 6.5° radius. Because of the very limited number (~ 125) of 'source' counts, the existence of a nuclear γ -ray line at 1.1 MeV in B10 cannot here be conclusively established. If a line is indeed the physical cause of our spectral feature, the ^{44}Ca , which emits γ radiation at 1.156 MeV following the decay of radioactive ^{44}Sc , is a likely candidate. This nucleus arises from ^{44}Ti according to explosive models of supernova nucleosynthesis^{5,6}, and was predicted by Clayton *et al.*⁷ to be the most likely galactic γ -ray spectral line to be discovered. Only supernova remnants less than a few years old should emit more intense γ -ray lines. The 1.6-MeV line flux inferred from our data equals the predicted flux⁸ for a supernova at a distance of ~ 3 kpc and an age of $\lesssim 100$ yr. These numbers do not seem unreasonable, considering that the ^{44}Ti yield 'per average supernova' need not characterise the actual yield of an unknown event in the Galaxy.

In any background runs, 2.6σ departures from a fitted continuum will occur, merely from random fluctuations. It is at least a remarkable double coincidence, however, that just the theoretically-predicted spectral anomaly occurred just when the background measurements crossed the galactic plane. We do not claim that positive evidence for nucleosynthesis in explosive galactic events has been detected, but we report these results in the hope that γ -ray astronomers will be stimulated to search the B10 portion of the Galaxy for the 1.16-MeV line with more sensitive equipment. If it does arise from ^{44}Ti , the associated ^{44}Sc -decay flux will be almost constant over our lifetimes.

If ^{44}Sc is responsible for the emission near 1.16 MeV, then a flux of comparable intensity is expected near 1.511 MeV, from positron annihilation. The B10 flux measured in the 0.44–0.52 MeV interval (which includes the positronium-decay spectrum⁹), is 5.1×10^{-4} photons $\text{cm}^{-2} \text{s}^{-1}$, which is within 1σ of the contribution from the power law in this interval. Evidently the available data are consistent with the presence of radioactive ^{44}Sc , but are insufficient to establish the existence of this species within the field of view.

Optical and radio data are inconclusive regarding the model of a supernova remnant as the source of the spectral anomaly. An optical supernova remnant has been identified by Van den Bergh *et al.*¹⁰ at galactic coordinates of 352.05° and $+0.13^\circ$, where there is an indication of faint filamentary structure together with an extended H II region. At 408 MHz, the remnant has an angular diameter of $30'$, which implies, for a 3-kpc distance, that the age is $\gg 50$ yr. This makes the remnant probably too old to be responsible for the observed anomaly. A much smaller source of 408-MHz radiation has been identified¹¹, at galactic coordinates 345.34° and $+1.43^\circ$. Although no supernova remnant has been identified optically at these latter galactic coordinates, the source can be considered a more likely candidate for the cause of the B10 anomaly, since its absolute minimum age is only ~ 10 yr.

Further observations of this region of the Galaxy should be made using more sensitive γ -ray spectrometers: they may confirm an important step forward in understanding the origins of elements.

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The role of Lyman- α photons in the interstellar medium

LYMAN- α 1,216 Å photons may be produced in a variety of astrophysical situations. These photons are lost through two-photon emission on interacting with hydrogen gas, and through absorption by interstellar grains, but velocity differences between the regions of production and destruction of these photons renders their destruction by the two-photon emission process relatively inefficient¹. Grain absorption is therefore the primary loss mechanism, but since it occurs over path lengths $\gtrsim 50$ pc, there are significant interstellar Ly- α fluxes. An early theoretical estimate¹ of the interstellar flux of diffuse Ly- α photons yielded a value $\sim 6.4 \times 10^{-4}$ erg cm⁻² s⁻¹ sr⁻¹. This estimate agrees well with the first Mariner-5 and Mariner-6 observations of this flux by Barth^{2,3}, the later giving a value of $\sim 4.9 \times 10^{-4}$ erg cm⁻² s⁻¹ sr⁻¹. Later authors^{4,5} have queried the precise fraction of this observed radiation which is genuinely extra-solar, although a significant interstellar component of Ly- α flux is generally conceded. A value in the range $1-4 \times 10^{-4}$ erg cm⁻² s⁻¹ sr⁻¹ would appear consistent with the available data. We note that the energy flux in Ly- α at the lower limit of this range is already ~ 5 times the average continuum of diffuse radiation⁶ in a 100-Å band pass centred on $\lambda = 1,425$ Å. It is also ~ 10 times greater than theoretical estimates of the continuum flux in the same waveband^{7,8}. Such a large Ly- α flux is difficult to understand if it arises from the Lyman continuum of O-B stars. Münch's original estimate¹ was based on the integrated effect of stellar chromospheres, the Ly- α photons being produced by non-thermal processes. Other likely sources of non-thermal Ly- α radiation include production in interactions of stellar wind with the interstellar medium, supernovae remnants and O V regions such as indicated by recent Copernicus observations^{9,10}. Lyman- α photons are also expected to be formed in intercloud regions where the production of such photons provides the main cooling process. Here we tentatively adopt a Ly- α flux in the range $\sim (1-4.0) \times 10^{-4}$ erg cm⁻² s⁻¹ sr⁻¹ as being appropriate for the general interstellar medium. It is of interest that the energy density associated with the upper end of this flux range is $\sim 1.68 \times 10^{-13}$ erg cm⁻³, ~ 0.25 of the total energy density of starlight in the Galaxy. Furthermore, we note that this energy density is of the same order of magnitude as that appropriate for the galactic magnetic field, the turbulent motion of interstellar gas and the cosmic microwave background. We show here that this high Ly- α flux is of basic importance for the physics of the interstellar medium.

The electrostatic charge on grains is an important parameter which influences grain-plasma interactions, and also, probably, the heating rate of the interstellar medium. The mean equilibrium charge on grains for several likely grain materials is expected to be determined by a balance between photoejections caused by ultraviolet photons and the capture of ambient electrons¹¹⁻¹³. A mean grain potential of several tenths of a volt has been calculated¹² using the observed flux of continuum diffuse radiation in the waveband 10–13.6 eV, and for a range of interstellar cloud conditions. The mean equilibrium potential

(considering only a balance between photoejections and recombinations) is given by

$$\bar{e}\Phi = kT \left\{ \left[\int_{E_i}^{\infty} F_{\gamma} Q_{\text{abs}} dE_{\gamma} / n_e v_e S \right] - 1 \right\} \quad (1)$$

where $F_{\gamma} dE_{\gamma}$ is the radiation flux in the photon energy range E_{γ} , $E_{\gamma} \pm dE_{\gamma}$. Q_{abs} is the grain absorption efficiency, y is the photoelectric yield, n_e , v_e , T are the electron density, velocity and temperature, S is a sticking coefficient and E_i is the photoelectric threshold. The value of E_i is typically ~ 6 eV for metals (including graphite) and ~ 9 eV for insulators such as silicates. In both cases these threshold energies are less than the Ly- α photon energy, $E_{\alpha} \approx 10.2$ eV. The photoelectric yield for small particles is a factor ~ 3 higher than that for bulk material¹². Taking this factor into account we adopt representative values of 0.1 for silicates and 3×10^{-3} for graphite using data of Willis *et al.*¹¹, for photon energies close to 10.2 eV. For the range of interstellar diffuse Ly- α flux adopted here the first term within brackets in equation (1) is changed by a factor of $\sim 0.4-4.3$ from its value assuming only a continuum ultraviolet flux⁶ 2×10^{-7} erg cm⁻² s⁻¹ sr⁻¹ Å⁻¹. We then obtain a mean steady-state grain potential

$$\bar{\Phi} = \begin{cases} \left\{ \frac{0.1\xi y}{n_e} - 0.0086 \right\} & V, T = 100 \text{ K} \\ \left\{ \frac{\xi y}{n_e} - 0.86 \right\} & V, T = 10^4 \text{ K} \end{cases} \quad (2)$$

where ξ is in the range 1.7–6.8 adopting $S = 1$, with the proviso that $\bar{\Phi}$ cannot exceed Φ_{max} given by

$$e\Phi_{\text{max}} \approx E_{\alpha} - E_i$$

The values of Φ_{max} for silicates and graphite are respectively ~ 1.2 and 4.0 V for the assumed values of E_i . Setting $n_e = 10^{-2}$ cm⁻³ we obtain the following values of $\bar{\Phi}$: for $T = 10^3$ K, the potential for silicates is ~ 1.2 V and for graphite 0.04–0.20 V; while for $T = 10^4$ K, for silicates it is ~ 1.2 V, and for graphite -0.35 – 1.12 V. These calculations involved only an equilibrium between photoejection and recapture of electrons by grains. A more rigorous determination of $\bar{\Phi}$ should take account also of the capture rate of positive ions. Such a procedure does not change our estimates of $\bar{\Phi}$ for silicates. For graphite, however, slight departures occur, but these are of negligible relevance in the ensuing discussion.

For silicate grains with a relatively high photoemission yield the mean potential saturates at ~ 1.2 V in both H I clouds and intercloud regions. The close equality of this potential to $(E_{\alpha} - E_i)/e$ would preclude a significant heat source from ejected photoelectrons if all grains acquired the same potential. A stochastic distribution about a mean grain potential is, however, expected to occur¹⁴, and the r.m.s. energy of emitted photoelectrons has a non-zero value which we estimate as $E_e \approx 0.5$ eV. The heating rate Γ_a from photoelectron emission from silicate grains by Ly- α photons is

$$\Gamma_a = \frac{\sigma n_g}{n_H} Q_{\text{abs}} y E_e F_{\alpha} n_H \text{ erg cm}^{-3} \text{ s}^{-1} \quad (3)$$

where σ is the geometrical cross section, Q_{abs} is the absorption efficiency of grains, n_g is the grain density, n_H is the hydrogen density, y is the photoelectron emission efficiency, E_e is the

mean energy of emitted photoelectrons and F_α is the flux of Ly- α photons. For $y = 0.1$, $E_c = 0.5$ eV we obtain

$$\Gamma_\alpha = (0.38-1.5) \times 10^{-26} \left\{ \frac{\sigma n_g Q_{abs}}{n_H (6 \times 10^{-22} \text{ cm}^2)} \right\} n_H \text{ erg cm}^{-3} \text{ s}^{-1} \quad (4)$$

for F_α in the adopted range.

With $\sigma n_g Q_{abs}/n_H \approx 6 \times 10^{-22} \text{ cm}^2$ (ref. 15) we get from equation (4) a heat input rate which is significantly higher than the corresponding rate calculated for photoemission by ultraviolet continuum photons. For graphite grains with $|\Phi| \lesssim 1.0$ V (in both cloud and intercloud regions, $y = 3 \times 10^{-3}$ and $E_c \sim 4$ eV) we find

$$\Gamma_\alpha = (0.1-0.36) \times 10^{-26} \left\{ \frac{\sigma n_g Q_{abs}}{n_H (6 \times 10^{-22} \text{ cm}^2)} \right\} n_H \text{ erg cm}^{-3} \text{ s}^{-1} \quad (5)$$

Again with $\sigma n_g Q_{abs}/n_H \approx 6 \times 10^{-22} \text{ cm}^2$ we obtain a heating rate which is consistent with available data on gas temperatures and cooling rates in both clouds and intercloud regions. The observed high flux of Ly- α photons could thus provide a major source of heating for the interstellar medium through their photoelectric effect on grains.

The line-centre photons of Ly- α cannot ionise hydrogen atoms, which have an ionisation threshold wavelength of 912 Å. Because of the natural width of the emitted and scattered line photons, there would be photons with energies > 13.6 eV in the line wing which could contribute to the ionisation rate of the interstellar gas. We can show, however, that the contribution of this process is not very significant compared with other mechanisms which have been proposed unless the Ly- α flux is equal to or higher than the larger value adopted in the preceding discussion.

We conclude that the recently observed large flux of diffuse galactic Ly- α photons would have an important effect in controlling physical processes in the interstellar medium. In particular the electric charge on grains, particularly silicate grains, will be primarily determined by photoejections caused by Ly- α photons. The heating of the interstellar medium will also mainly be caused by this process. If external galaxies emit an equally high fraction of their luminosity in Ly- α radiation as is indicated in our own galaxy, their effect on the intergalactic medium as well as their possible cosmological role will need to be considered. A more precise determination of the Galactic Ly- α flux is clearly a matter of paramount astrophysical importance.

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Primitive grain clumps and organic compounds in carbonaceous chondrites

We show here that the physical conditions in prestellar molecular clouds favour the condensation of complex organic polymers, including amino acids, within a matrix of smaller refractory particles. Such composite grain clumps with dimensions exceeding 1 μm could be expelled along with gaseous material in protostellar cocoons, causing the widespread dispersal of biological activity in the Galaxy. We argue that grain clumps of the type considered here may be identified with μm -sized inclusions in carbonaceous chondrites.

Carbonaceous chondrites, with a carbon content of several per cent mainly in the form of aromatic polymers, and including amino acids in trace quantities, are generally believed to be among the most primitive solid bodies in the Solar System. The compaction of mineral particles with a substantial admixture of trapped volatiles must have occurred at temperatures in the range 350–500 K, with no subsequent reheating above ~ 500 K (refs 1 and 2).

Several striking isotopic anomalies have been discovered in mineral separates from carbonaceous chondrites^{3–7}. Such anomalies have tentatively been attributed to inclusions of interstellar grains which condensed in novae or supernovae explosions^{3,5,8,9}. This explanation is consistent with the occurrence of heavily irradiated μm -sized mineral separates rich in ²²Ne in the Orgueil meteorite¹⁰. The presence of μm -sized inclusions, each comprised of closely packed aggregates of grains of 100 Å (ref. 11), is also suggestive of interstellar grain clumps within carbonaceous chondrites.

An understanding of the origin of carbonaceous chondrites may have an important bearing on the early history of the solar nebula, and in particular on theories of planetary formation. The efficient adhesion of relatively cold refractory grains (for example, graphite or silicate particles) in low velocity grain-grain collisions could occur if these grains possessed mantles composed of organic polymers which are adhesive at temperatures ~ 300 K. Such organic polymers have been tentatively identified by their infrared spectral features in cometary as well as interstellar dust^{12,13}.

We argue here that interstellar molecular clouds which are the most probable sites for the condensation of polymeric mantles around grains are also likely to provide suitable venues for the formation of composite grain aggregates, by the adhesion of such coated grains in grain-grain encounters. Such grain clumps of sizes $\sim 1 \mu\text{m}$ pre-existing in the solar nebula could have served as aggregation centres for the growth of carbonaceous chondrites, perhaps representing the earliest stage of planet formation.

Large molecular clouds with masses in the range $\sim 10^3$ – $10^6 M_\odot$ are widespread in the galactic disk. Such clouds, typified by W3, OMC-2, NGC2024, Sg B2, are generally believed to be progenitors of OB associations. In a typical extended cloud of diameter ~ 10 pc, observations of molecular CO at millimetre wavelengths leads to an estimate $n_{\text{H}_2} \approx 3 \times 10^3 \text{ cm}^{-3}$ for the smeared out hydrogen density¹⁴. More complex molecules, including HCN, H₂CO, tend to be more localised in their spatial distribution, generally associated with infrared knots, OH masers and presumably protostellar clouds. Molecular densities in such clouds are difficult to estimate. The requirement for collisional excitation of optically thin lines of H₂CO, HCN by neutral particles gives a lower limit $n_{\text{H}_2} > 10^5$ (ref. 14), but densities $\sim 10^6 \text{ cm}^{-3}$ or higher are most probably appropriate to protostellar clouds.

One may also argue that molecular clouds are not in a state of free-fall collapse¹⁵. Condensation may be slowed down by several processes, including effects of magnetic pressure, rotation and turbulence. We assume here that typical collapse times for an entire cloud, as well as for fragments within it, are of the general order of 10^6 yr. Such a condensation time, together

with the estimated total mass of protostellar clouds, gives a rate of star formation which is consistent with observations.

A molecular cloud fragment collapsing towards a protostellar situation will contain a mass fraction of $\sim 10^{-2}$ of refractory grains such as graphite, silicate and iron particles of mean radius $a_1 = 2 \times 10^{-6}$ cm. The first stages of collapse will be accompanied by accretion of organic molecules on to these grains. Since a significant mass fraction of C and O is initially in solid grains, the maximum extent of mantle growth is not likely to exceed 50% of the original radius. This gas phase accretion would proceed to effective completion on a time scale which is short compared with the estimated collapse time of $\sim 10^6$ yr. The grain radius may now be assumed to be 3×10^{-6} cm (50% increase) in accord with our earlier remarks. The precise composition of molecular mantles is uncertain, but a hybrid mixture of organic polymers is likely to ensue.

Refractory grains with such tar-like polymeric coatings tend to stick to one another in low velocity grain-grain collisions at temperature $T \approx 300$ K. Suppose $n_H (= 2n_{H_2})$ is the total hydrogen density and n_g is the grain density at this stage of protostellar collapse. Assuming an initial grain mass fraction of $\sim 1\%$, we have (for any reasonable grain specific gravity)

$$\frac{n_g}{n_H} \approx 3 \times 10^{-10} \quad (1)$$

The rate of growth of a grain clump of radius r by this process is given by

$$\begin{aligned} \frac{dr}{dt} &= \frac{\alpha n_g}{s} \left[\frac{kT \left(\frac{4}{3} \pi a_1^3 s \right)}{2\pi} \right]^{\frac{1}{2}} \\ &= \alpha n_g \left[\frac{2kTa_1^3}{3s} \right]^{\frac{1}{2}} \end{aligned} \quad (2)$$

where α is the sticking probability, s is the mean specific gravity of the grain clump material, $a_1 (= 3 \times 10^{-6}$ cm) is the radius of polymer coated grains, n_g is the number density of grains, and T is the kinetic temperature. We assume in equation (2) equipartition of energy between grains and gas and a Maxwellian distribution of grain velocities. Sticking of grains occurs by collisions during their Brownian motion with relative speeds of ~ 10 cm s $^{-1}$. With $\alpha \approx 1$, $T \approx 300$ K, $s = 1$, $a_1 = 3 \times 10^{-6}$ cm and using equation (1) we obtain

$$\frac{dr}{dt} \approx 8.2 \times 10^{-18} n_H \text{ cm yr}^{-1} \quad (3)$$

In the available time, $\sim 10^6$ yr, we obtain clump diameters $2r \sim 1 \mu\text{m}$ for a typical value of the molecular density $n_{H_2} \approx 3 \times 10^6$ cm $^{-3}$. Larger clumps could arise from higher density regions.

The ultimate dispersal of a protostellar cocoon, including large grain clumps, may have a role in the removal of angular momentum from a central protostellar condensation, thus permitting further contraction and evolution on to the main sequence. A large fraction of composite grain clumps in such cocoons could probably survive the 'switching on' of the stars in an OB association, and they may be carried along with systematic gas flows into the general interstellar medium. Such grain clumps could indeed constitute an appreciable fraction by mass of all interstellar dust.

Large grain clumps of the type discussed here in a protoplanetary disk could also serve as accretion sites for smaller grains which condense within the disk, the process leading to the formation of planetisimals in the first instance, and eventually to planets. With a minor degree of metamorphism, such objects at

an intermediate stage of aggregation would seem to resemble the carbonaceous chondrites.

It is tempting to speculate that amino acids which have been discovered in carbonaceous chondrites¹⁵⁻¹⁷ had their origin in presolar grain clumps of the type considered here. The formation of simple amino acids (for example, glycine) is expected to take place in dense interstellar molecular clouds which may well be the cradle of life. The possible precursors of glycine, namely formic acid (HCOOH) and methanimine (CH₂NH), have already been observed in dense molecular clouds, and the reaction



leading to the production of glycine is known to be exothermic. It may be relevant that glycine is the most abundant of the amino acids detected in chondrites¹⁷. Amino acids of this type, and of greater complexity may be trapped in the tarry polymeric component of our grain clumps, and be dispersed throughout interstellar space, being securely protected from destruction by ultraviolet photons by the matrix of smaller refractory grains in which they are embedded.

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Comparative ionospheric and plasmaspheric electron contents from three world regions

THE radio beacon experiment¹⁻³ aboard the geostationary Applied Technology Satellite ATS-6 enables the determination of total electron content (TEC) to be made by two independent methods. The first—the Faraday polarisation rotation technique—has been widely used in obtaining TEC data. Faraday rotation is dependent on the Earth's magnetic field, and, since its magnitude is heavily weighted near the Earth, it is considered to provide integrated electron content values for altitudes below $\sim 1,500$ km. The second—the dispersive-group-delay technique, in which the phase of the modulation envelope between a carrier and its sideband is compared at two frequencies—is independent of the Earth's magnetic field and thus yields the integrated electron content between the observer and the satellite signal source. The Faraday content, N_F , and the dispersive-group-delay content, N_T , therefore, yield the TEC up to $\sim 1,500$ km and geostationary altitudes, respectively. The difference between N_T and N_F yields the content above $\sim 1,500$ km, which is referred to as the plasmaspheric content, N_P .

The ATS-6 satellite was launched into a geostationary orbit in May 1974. During phase I of its operation, when

the satellite was located at 94°W, the US Army Electronics Command operated observation stations at Fort Monmouth, New Jersey (40.18°N, 74.06°W); Richmond, Florida (25.60°N, 80.40°W); and Sao Paulo, Brazil (23.50°S, 46.50°W). In May 1975, the satellite was moved to 35°E for phase II of its operation. During this phase, the observation stations were moved to Haifa, Israel (32.87°N, 35.09°E) and Kiruna, Sweden (61.84°N, 20.41°E). In August 1976, the satellite was moved to its final parking position at ~134°W, with some of the observation stations being reactivated and others being established.

The Faraday observations were made with the 140-MHz satellite beacon emissions. The dispersive-group-delay obser-

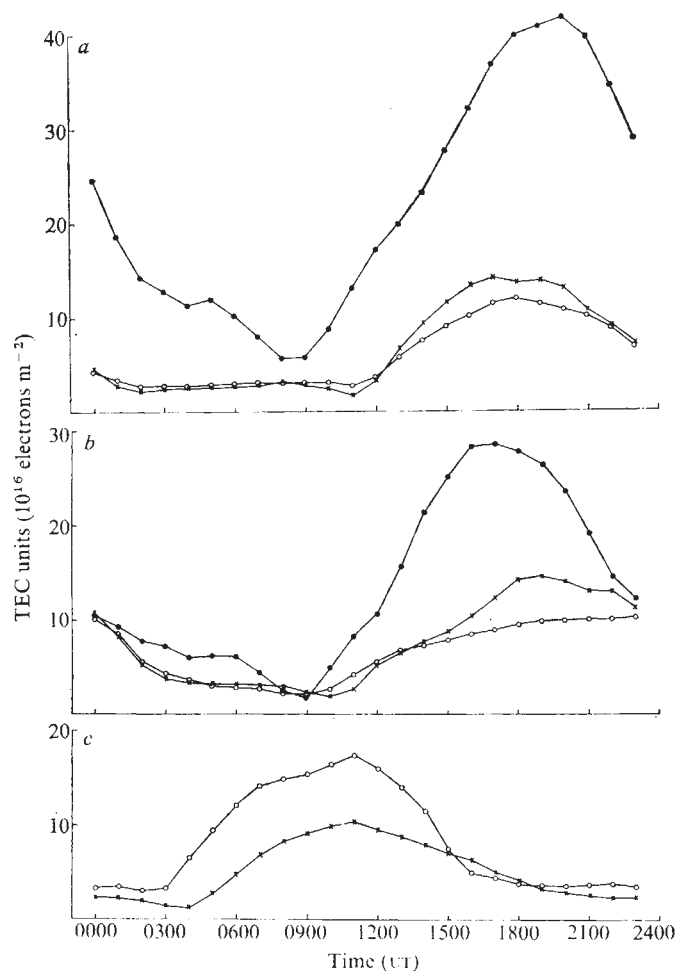


Fig. 1 *a*, Hourly mean monthly variation of N_F at Sao Paulo, (●) (LT = UT - 3); Fort Monmouth, New Jersey (○) (LT = UT - 5); and Richmond, Florida (×) (LT = UT - 5), during February 1975. *b*, Same as *a*, but for May 1975. *c*, Hourly mean monthly variation of N_F at Kiruna, Sweden (×) (LT = UT + 1); and Haifa, Israel (○) (LT = UT - 2), October 1975.

vations were made with the 140 and 360-MHz beacons modulated with sideband displacement of $\Delta f = +1$ MHz.

Figure 1*a* and *b* depict a comparative mean monthly variation of TEC measured by the Faraday technique at Fort Monmouth, Richmond, and Sao Paulo for February 1975 and May 1975, respectively. The solar zenith angle dependence of TEC is shown in the relative values of TEC recorded at the two mid-latitude stations in the Northern Hemisphere. The comparatively higher TEC values recorded at Sao Paulo, even if seasonal effects are ignored (compare Fig. 1*a* and *b*), were due to the passage of the ray path from satellite to observer through the Equatorial Anomaly region. At equatorial regions, ionospheric plasma which moves upwards from an imposed east-west electric field diffuses downwards again, but obliquely, along the geomag-

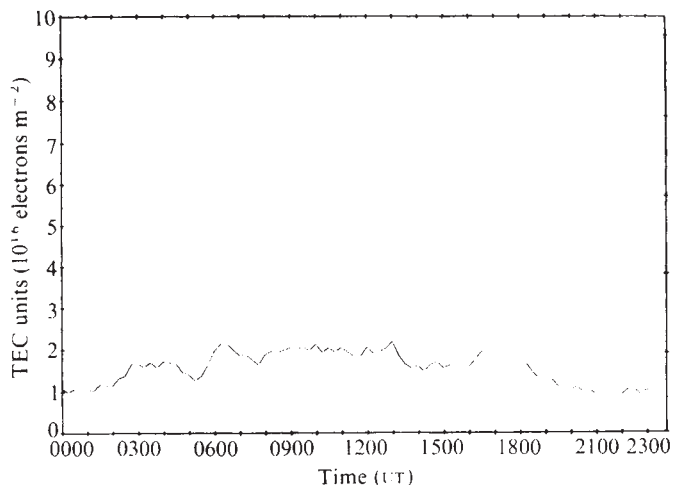


Fig. 2 Mean monthly variation of N_F at Kiruna, Sweden (at 15-min intervals), October 1975.

netic lines of force. Instead of returning to the source, the plasma arrives at two places—one to the south and one to the north of the equator. The electron content is then depleted at the magnetic equator, but is enhanced at the anomalous regions. The relatively higher TEC values for February as compared to May, confirm topside sounder results⁴ that the anomaly content crests are larger in the summer hemisphere. In addition, the ray path was in the vicinity of the South Atlantic Anomaly region where the magnetic field is abnormally weak and the loss of radiation belt particles is rapid. TEC enhancements are attributed to ionisation produced by the precipitating energetic trapped electrons that mirror low in the anomaly^{5,6}.

The comparative variation of TEC at a mid-latitude station and at a station near the auroral zone is shown in Fig. 1*c* for October 1975. The consistently higher TEC values at Haifa were due to the influence of the solar zenith angle on TEC. The passage of the ray path from Kiruna to the satellite through the density trough^{7,8} was also responsible for the observed depressed TEC values.

The mean monthly variation of the plasmaspheric electron content at Kiruna, Sweden, for October 1975 is shown in Fig. 2. The maximum content was during the day and the minimum at night, with average values between ~1.0 and ~2.0 TEC units.

Finally, the comparative variation of the plasmaspheric electron content, N_P , at Fort Monmouth and Sao Paulo during May 1975 is shown in Fig. 3. In general, the mean monthly values of N_P at both stations lay between ~1 and ~2 TEC units. The mean diurnal variation at Sao Paulo indicates that N_P had higher values during the day than during the night. The times of N_P enhancement were close to those at which the equatorial anomaly is formed and maintained⁴, and the time of N_P depression corresponded to

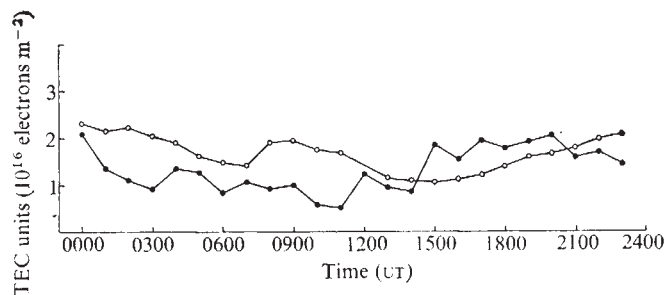


Fig. 3 Mean monthly variation of N_P at Fort Monmouth, New Jersey (○); and Sao Paulo, Brazil (●), May 1975.

disappearance of the anomaly. It is therefore possible, that during the formation of the anomaly and during its maintenance, the plasmaspheric and the ionospheric electron densities are increased.

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Meridional interactions between rainfall and surface pressure

WE have been investigating temporal variation in rainfall over a part of the Southern Hemisphere^{1–4}. In view of the current concern regarding food production and future climatic change (for example, a shift in the westerlies over the Northern Hemisphere) this area of research is of some importance. The recent drought and resulting famine in the Sahel^{5–7} also shows that stresses in one hemisphere can be a burden on the food resources of the other. Until now, however, quantitative interactions between rainfall and the general circulation have not been given; here we show first, that there are structural relationships between annual rainfall over Southern Africa and the month to month variation in the mean latitude of the surface high pressure belt, L (ref. 8). The evidence adds credibility to findings related to the temporal variation in rainfall^{2,4}. Second, a means of forecasting annual rainfall totals is shown to be feasible. The method used in the analysis is neither geographically restricted, nor applicable to rainfall only.

A linear step-wise regression analysis was first carried out on 30 yr of rainfall data and the associated value of L (1942–71). In this way the independent variables, L , can be quickly assessed for their importance in predicting rainfall. The analysis was carried out on the total annual rainfall for the regional temporal series A as defined in refs 2 and 3. This time series represents the rainfall over an area whose middle is at $\sim 27^\circ\text{S}$.

We will give the months in the order they were taken into the regression. When the total rainfalls were regressed on the monthly L values, January and September were included. The fit of the regression is significant at the 2.5% level. To check for some sort of organisation in rainfall totals unaccounted for by the regression, a plot of the residuals was carried out. There is evidence of an oscillatory pattern in the residuals, the period being 2–3 yr. This may

be related to the quasi-biennial oscillation which is now a well accepted geophysical phenomenon⁹.

A regression of rainfall totals on L values during months in the preceding year resulted in the inclusion of June and March. The fit is significant at the 1% level. A plot of the residuals again indicates a wave-like pattern with period in the range 2–3 yr.

Mean latitudes, L , for one year, together with those for all months in the previous year, were then included in the regression, bringing the L -value for January of the year in question as well as June, March and February of the preceding year. The fit of this regression was significant at the 1% level. The predictors are not just the addition of the two sets from the previous regressions, which results from the use of the multiple correlation coefficient between rainfall and L (ref. 10).

As a forecasting equation this particular grouping of variables accounts for 52% of the total variance in annual rainfall. This is an encouraging result. A plot of the residuals against time indicated that a linear term was required in the regression. Such a term was added to the set of predictor variables and the analysis repeated, with the linear trend as the fifth term, or that of least importance. The

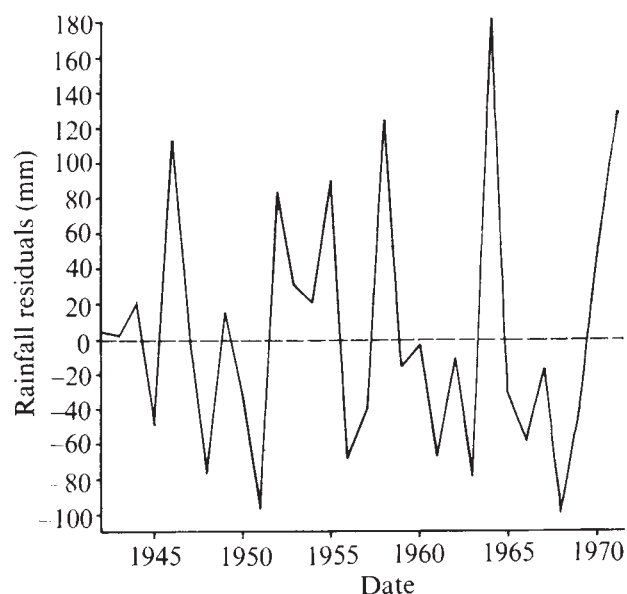


Fig. 1 Showing the rainfall residuals in a regression on the mean latitude of the surface high pressure belt for January in a current year, and June, March and February of the preceding year; the linear trend has been removed.

total variance now accounted for 58% of the whole. The residuals still exhibited an oscillatory pattern (Fig. 1) and they were therefore submitted to spectral analysis. Because of the short length of the series (30 terms) Burg's maximum entropy method was used¹¹. The spectrum shows excessive variance associated with periods of 5.88, 3.03 and 2.22 yr (Fig. 2).

A trigonometric function was fitted to the residuals, and resulted in the use of periods of 6.0 and 3.0 yr. The shortest period was considered too weak to include in the equation. The additional variance accounted for by this step was significant at the 1% level. In all, just over 84% of the total variance in rainfall has been accounted for, and the standard errors for forecasts is 6 cm, which is quite acceptable.

The final forecasting equation therefore contains as predictor variables:

(1) The mean latitude of the surface high pressure belt

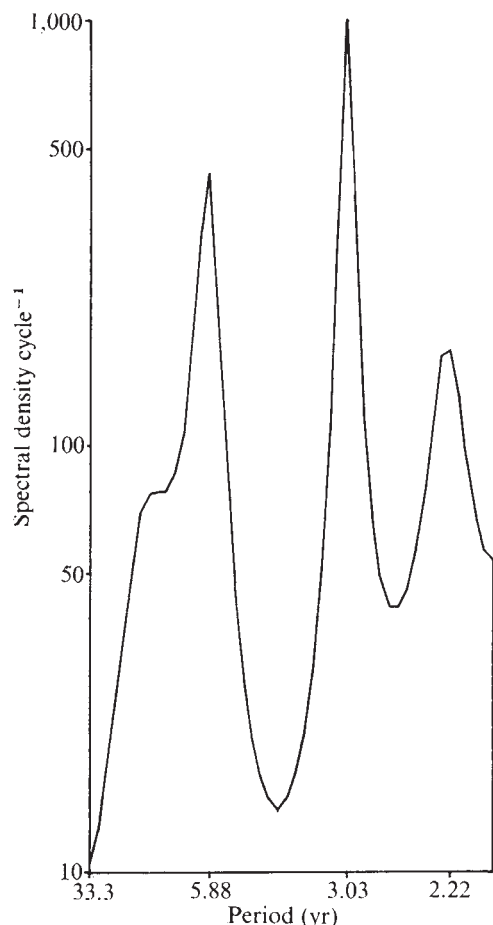


Fig. 2 Showing the spectrum of residuals when annual rainfall totals are regressed on the mean latitude of the surface high pressure belt for January in a current year, and June, March and February of the preceding year.

for January of a current year, with regression coefficient equal to -15.24 .

(2) As above, but for June, March and February of the preceding year, with regression coefficients -9.31 , 19.41 and 13.96 respectively.

(3) A linear term in time with regression coefficient 3.69 .

(4) A constant term equal to -422.82 .

(5) A trigonometric function having periods of 6.0 and 3.0 yr with cosine and sine coefficients 7.28 and -49.03 , and -47.82 and 39.04 respectively.

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"Upper Jurassic" sediments of South Africa

MARINE sedimentary rocks exposed near Knysna in the Cape Province of South Africa were assigned to the Upper Jurassic on the basis of a fragment of an ammonoid and seven previously undescribed species of ostracod¹. A more thorough examination of the microfossils in these rocks indicates that this assignment was erroneous and that the beds are of Early Cretaceous, probably Hauterivian, age². This age determination was based on identification of fifty species of palynomorph, five species of calcareous nannofossil, and twelve species of foraminifera. Both the palynomorphs and the foraminifera indicate a correlation with the upper part of the Sundays River Formation, a unit reliably dated as Valanginian to Hauterivian. The nannofossils indicate a Valanginian or Hauterivian age.

Ordinarily, a slight error in the age of a few small exposures in a remote portion of the globe would not be a matter of significance. These were the only marine sedimentary rocks of Jurassic age known from South Africa, however, and their age was considered to be important for timing the break-up of Gondwanaland³. In a very superficial search of the literature, we noted numerous citations of the Jurassic age (see refs 3-7). It was therefore thought desirable to bring to the attention of those concerned with the problems of Gondwanaland rifting that there are no marine sedimentary rocks of known Jurassic age in South Africa.

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Cyanophycean nature of stromatoporoids

STROMATOPOROIDS are calcareous fossils abundant in many Palaeozoic (mainly Silurian and Devonian) shallow-water carbonate sediments. Forms ascribed to stromatoporoids have also been reported from Mesozoic epicontinental and tethyan carbonate deposits¹. The nodular, tabular or cylindrical and, generally, vertically laminated calcitic skeletons of stromatoporoids (Fig. 1a), ranging in size from a few mm to >1.0 m, are internally formed of curved plates, short or long pillars and vertical walls. A characteristic feature of many stromatoporoids are stellate structures, a few mm to 6-7 cm across, termed astrorhizae. In spite of 150 yr of investigation the systematic position of stromatoporoids is still controversial. They have been variously interpreted as foraminifers, sponges and hydrozoans^{2,3} with discussion on the latter two groups bearing particularly on the interpretation of the skeletal morphogenesis^{4,5}. New discoveries⁶ of peculiar modern sponges with mixed calcareous and spicular siliceous skeletons (Sclerospongiae) reopened discussion on the relationship of this group to stromatoporoids, initiated at the beginning of the century by Kirkpatrick^{7,8}. Hartman and Goreau's suggestion⁹ has been taken up and supported by Stearn^{5,10} and Wendt¹¹. Stearn argued that stromatoporoids should be recognised as a separate subphylum of the Porifera. Wendt similarly regarded stromatoporoids as

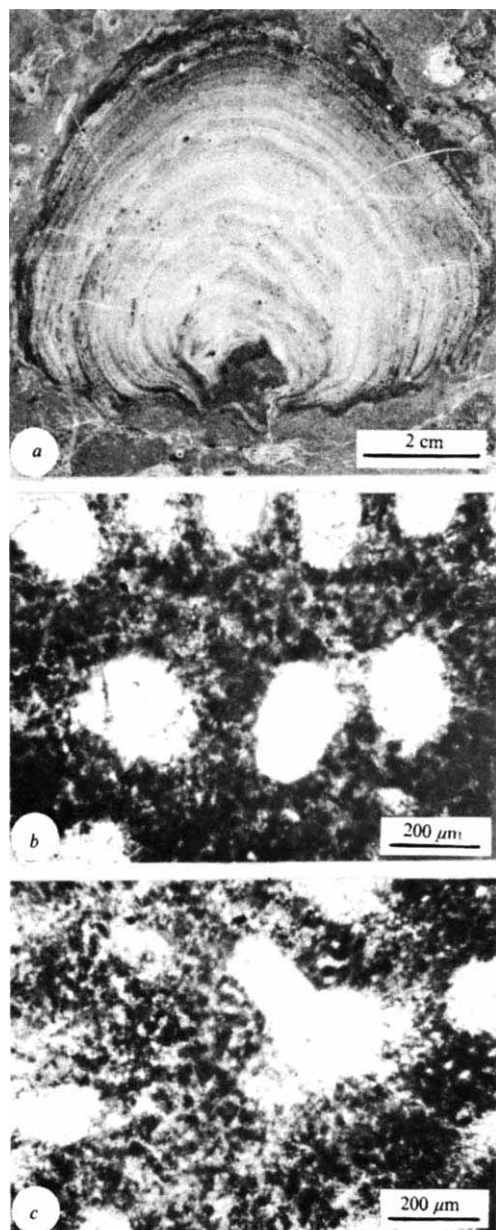


Fig. 1 *a*, Vertical axial section of the distinctly laminated skeleton of *Stomatopora undata* Riabinin from the Upper Devonian of central Poland; *b*, *c*, enlarged fragments of the same skeleton in vertical (*b*) and tangential (*c*) section showing granular microfabric representing permineralised aggregates of coccoidal cells, transmitted light.

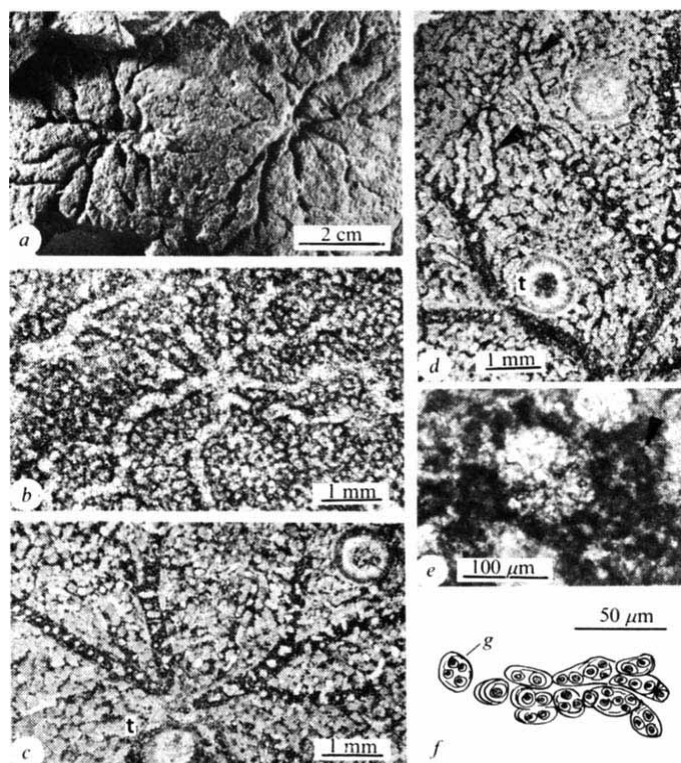
a conservative order of the Calcispongiae closely related to the Pharetronida. Spicules, however, are quite unknown in stromatoporoids and the discovery of fossil (Cretaceous) sponges with preserved spicules^{12,13} shows that their absence cannot arise from diagenetic dissolution as Hartman and Goreau suggested. Interpretation of the astrophorizae is critical to any discussion of stromatoporoid affinities. As poriferans, the astrophorizae become traces left by the sponge exhalant canals, or as hydrozoans they are structures homologous to coenosarcial stolons (hydrophorizae). Kaźmierczak¹⁴ rejected both interpretations and argued that astrophorizae are traces of foreign organisms probably commensal or mutualistic with stromatoporoids. The new observations reported here modify but confirm this.

Discovery of unusually well preserved astrophorizae reveals the nature of stromatoporoids as permineralised colonies of coccoidal cyanophytes homologous with modern and fossil

stromatolites. The astrophorizae occur in *Parallelopora* aff. *planulata* (Hall and Whitfield) obtained from the Upper Devonian (Frasnian) limestone of the borehole Niesiołowiec IG-1 (south-eastern Poland) at a depth of 1,330–1,336.4 m. In contrast with previously known astrophorizae, which appear either as a stellate system of furrows on the surface of the skeleton (Fig. 2*a*) or as radiating tubes with rare cross partitions filled by sparry calcite in thin section (Fig. 2*b*), the astrophorizae in *P.* aff. *planulata* are almost wholly filled by granular calcareous material (Fig. 2*c*). In transmitted light this is distinctly darker than the surrounding skeleton against which it sharply abuts. At many points, however, the skeleton passes into the dark astrophorizal infilling. The structural uniformity of the astrophorizal infilling and the surrounding skeleton is also stressed by the similar size and outline of the interskeletal spaces in both tissues. Where the dark granular material is absent the astrophorizal canals are filled by spar as is generally the case (Fig. 2*d*, arrowed). Under higher magnification the clotty infilling of the astrophorizae can be recognised as aggregations of spherical and subspherical bodies, slightly darker peripherally. Individual bodies have an average diameter of 10–40 μm and are distributed irregularly, rarely forming short pseudofilamentous rows (Fig. 2*e*, arrowed).

The globular bodies from the astrophorizal infillings and the granules of the surrounding skeleton are essentially similar, the main difference lying in the higher crystallinity of the

Fig. 2 *a*, Large astrophorizae on the surface of the Middle Devonian stromatoporoid *Trupetostroma* sp.; *b*, astrophorizae in tangential thin-section of Silurian *Plectostroma* sp. showing tubes filled by sparry calcite, transmitted light; *c*, *d*, tangential thin-sections through astrophorizal tubes in the Devonian *Parallelopora* aff. *planulata* (Hall and Whitfield) filled by dark granular material which is locally absent (arrowed), transmitted light. (Note also cross sections (t) of intergrown syringoporoid tabulate corals); *e*, enlarged fragment of astrophorizal infilling showing subcircular outlines of permineralised common sheaths of aggregates of coccoidal cells forming rare, short chains (arrowed), transmitted light; *f*, a colony of the extant *Entophysalis samoensis* Wille built of *Gloeocapsa*-like aggregates of cells (g) enclosed in common sheaths (after Wille).



latter, which results in their higher transparency and indistinct outlines. Granular microfabric is common in stromatoporoids and has been referred to as cellular, melanospheric or maculate microstructure¹⁵ (Fig. 1b and c).

The spheroidal bodies composing the infillings of astro-rhizae can be identified as remnants of permineralised aggregates of cells of coccoid cyanophytes. Identical structures have been observed in modern calcareous stromatolites formed by entophysalidacean blue-green algae^{16,17}, where they form colonies composed of numbers of cells arranged in pseudofilamentous strands (*Entophysalis*) or are distributed irregularly^{18,19}. Such cells, surrounded by mucilage sheaths, are usually found within greatly enlarged common mucilage sheaths enclosing *Gloeocapsa*-like aggregates (Fig. 2f, g). It has been only recently observed¹⁷ that during permineralisation with calcium carbonate it is the tough outer sheaths which envelop particular aggregates of cells that are most resistant, and commonly remain as the only trace of the primary structure of a colony. Similarly, resistant sheath material has been observed in Late Precambrian pleurocapsalean cyanophytes from silicified dolomite²⁰. The filamentous pattern of astrophthalic canals is reminiscent of linear cell masses of some siphononematacean cyanophytes during the stigonematoides growth stage²¹. The granular microfabric of the astrophthalic and the surrounding skeleton indicates that the same or very similar cell aggregates took part in the formation of both.

There are two questions related to the last statement: first, why, having the same microstructural character as the surrounding skeleton, the astrophthalic infilling is darker and less crystalline, and second why the astrophthalic are usually found empty? An explanation to the first question may lie in the colony organisation of extant coccoid cyanophytes in which *in situ* germination of gonidia, endo- or exospores or even groups of nannocytes leads to the formation of new colonies continuing growth as an integral part of the old colonies^{18,19,22}. *In situ* germination of spores is particularly common in some pleurocapsalean cyanophytes²⁰. There is little doubt that astrophthalic represent such *in situ* developed new coccoid colonies, but with significantly reduced permineralisation potential compared with the parent colony. The modern counterpart of the stellate pattern of astrophthalic canals can be found in radially filamentous juvenile stages of such colonial coccoid cyanophytes as *Oncobysa rivularis* or *Pleurocapsa minor*²¹. As in astrophthalic, the filamentous aggregates of cells in these forms grow first more or less parallel to the substratum branching dichotomously or trichotomously and then, as a result of tetrachotomy, grow upwards losing their filamentous habit. Empty astrophthalic canals represent weak or absent permineralisation of the young colonies, insufficient to protect the organic matter against decomposition. Differences in the degree of permineralisation of different growth stages within the same coccoidal strains means active control of the mineral precipitation at least by the young colony. More detailed studies on the organisation and mode of formation of modern coccoid cyanophytes, especially those encrusted by calcium carbonate, are needed before a complete explanation of stromatoporoid biology can be given.

Removal of the Stromatoporoidea from the animal kingdom to the Cyanophyta requires the complete re-evaluation of their taxonomy, palaeobiology and geological significance. This and a more extensive discussion of the new data presented here will follow in a forthcoming paper. The main conclusions are:

(1) stromatoporoids represent permineralised colonies of coccoid cyanophytes closely related to those associated with fossil and modern calcareous stromatolites (cyanophytic stromatoids), formed by *in situ* precipitation of calcium carbonate,

(2) stromatoporoid stromatolites are the Phanerozoic continuation of the Precambrian calcareous stromatolites in

which coccoid communities also had a considerable role^{23,24},

(3) stromatoporoid stromatolites with their internally differentiated structure, and clear evolutionary tendencies⁴ may contribute significantly to the systematics and correlative value of Precambrian stromatolites,

(4) the very high permineralisation of stromatoporoid coccoid cyanophytes is comparable only with calcium carbonate encrusted cyanophycean communities known from modern freshwater and brackish environments²⁵. This suggests that the epicontinental seas occupied by stromatoporoids had other than present-day salinities, a conclusion supported by the recently reported²⁶ *in situ* association of *Amphipora* with non-marine volvocacean algae.

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Palaeognathous bird from the Cretaceous of Central Asia

Two skulls of an unquestionably palaeognathous bird were found by the Polish–Mongolian Palaeontological Expedition to the Gobi Desert¹. Both specimens originate from the same locality, the beds being referred to as Barun Goyot Formation (?Campanian²). One skull, badly damaged, has been described as *Gobipteryx minuta* and the new order Gobipterygiformes has been allocated tentatively to the Palaeognathae³. Examination of the second, better preserved, although incomplete skull has now confirmed this allocation. Thus, *Gobipteryx* is the oldest known palaeognathous bird, the earliest hitherto recorded fossils of this group being of Eocene age⁴. I estimate the skull of *Gobipteryx* to be 45 mm long, so that the bird was roughly the size of a partridge.

Gobipteryx displays palaeognathous affinities both in the pattern of the palate and the features directly related to the ratite rhyndokinesis⁵ (Figs 1 and 2). Anteriorly the vomers are fused and closely approached by the maxillopalatines. At the rear, the vomers diverge gradually and articulate suturally with the pterygoids, which have a medial socket near the posterior end, obvious for a basipterygoid articulation. The palatine articulates suturally with the outer margin of the pterygoid, so that parasphenoidal contact is ruled out. As far as it preserved, the palate of *Gobipteryx* satisfies all the criteria of the palaeognathous type⁶. The most

striking rhynchokinetic feature is the anterior extension of the nasal opening together with the lack of the connection between the nasal and the maxilla. The free-ending, ascending process of the maxillopalatine, present in *Gobipteryx*, elsewhere occurs only in some ratites (*Rhea*, *Casuaris* and *Dromaius*). Moreover, the loose contact between the nasal process of the premaxilla and the premaxillary process of the nasal, clearly seen in *Gobipteryx*, occurs in all ratites and some members of the Tinamidae. The common occurrence of such a palatal and rhynchokinetic condition provides new evidence of the coupling of these two characteristics⁶.

In some features *Gobipteryx* seems to resemble the Australian ratites, in particular its palate structure is similar to that of *Casuaris*: the posterior bifurcation of the vomers is very deep, with their lateral edges lying more ventrally than the medial ones; the pterygoids widen distinctly in the region of the palatine articulation; the palatine articulates mostly if not exclusively with the pterygoid; the outer margin of the palatine is much thicker than the inner blade and projects ventrally. In *Gobipteryx* the palatine is posteriorly enclosed in the slight anterior bifurcation of the pterygoid, with the small outer tine projecting antero-laterad. Such a pterygoid bifurcation was thought to be unique in *Apteryx*⁷. Thus, *Gobipteryx* shares some characteristics with both *Casuaris* and *Apteryx*⁵ and unless it is

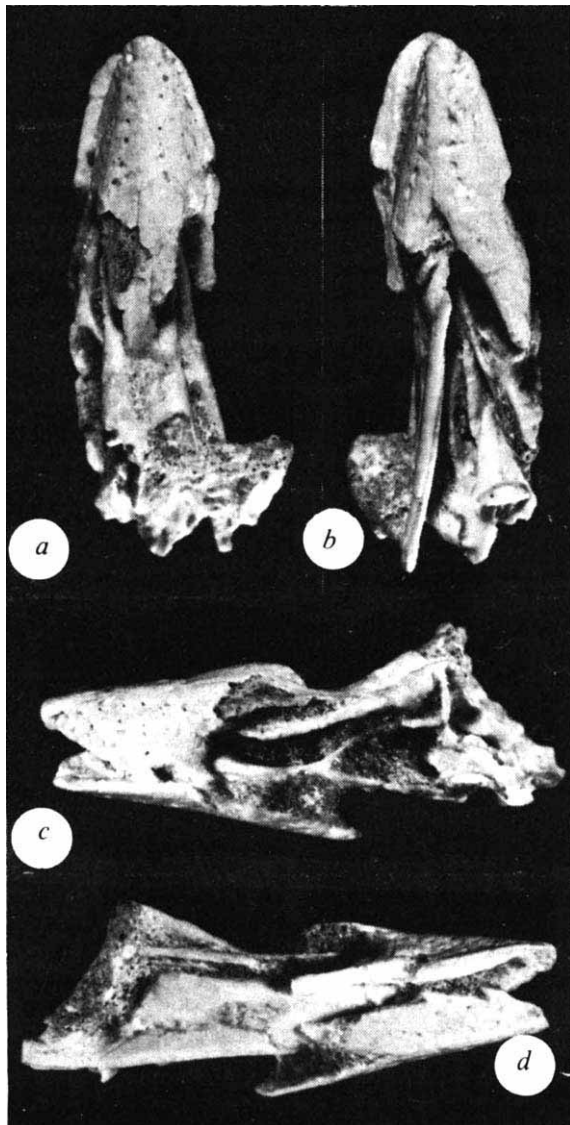


Fig. 1 *Gobipteryx minuta* Elzanowski 1974. The second skull. a, Dorsal view; b, ventral view; c, left lateral view; d, right lateral view ($\times 2.5$).

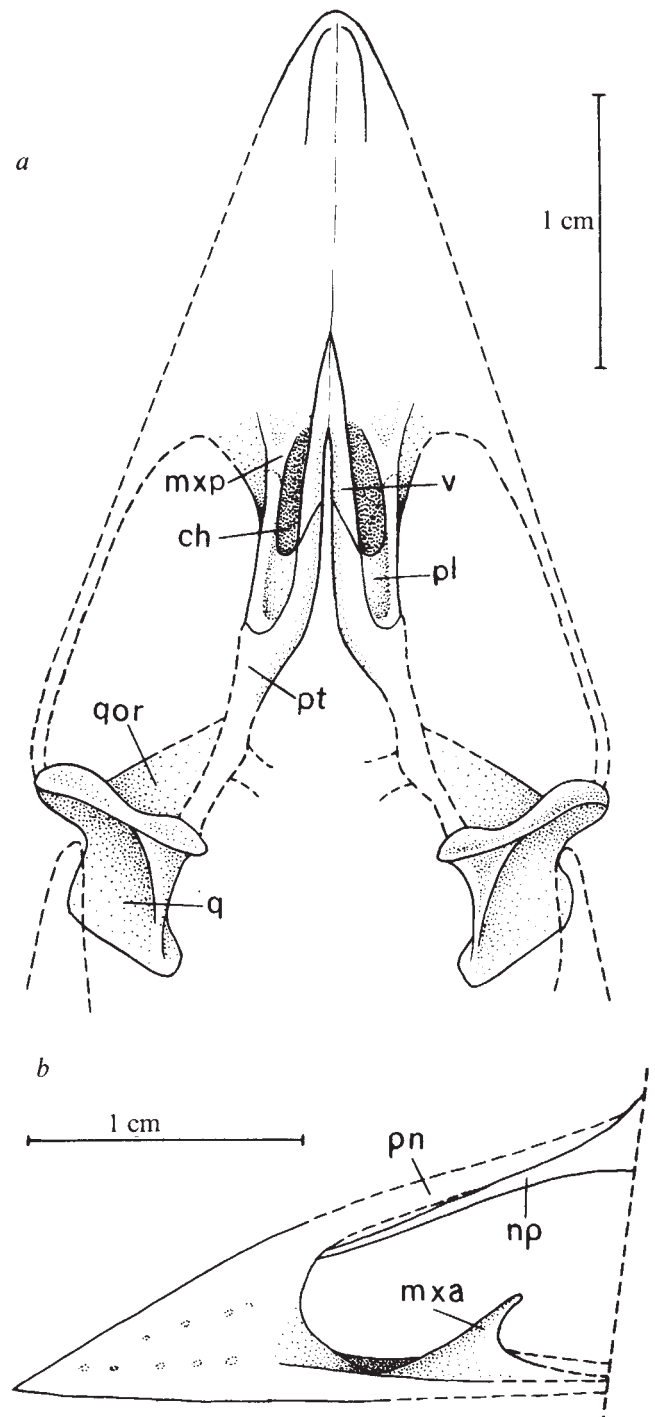


Fig. 2 *Gobipteryx minuta* Elzanowski 1974. Partial restorations of the skull. a, Ventral view; b, rostral part in lateral view. ch, internal naris; mxa, ascending process of the maxillopalatine; mxp, maxillopalatine; np, premaxillary process of the nasal; pl, palatine; pn, nasal process of the premaxilla; pt, pterygoid; q, quadrate; qor, orbital process of the quadrate; v, vomer.

assumed that these are primitive characteristics of the Palaeognathae as a whole, they might indicate a closer relationship between the Casuariformes and Apterygiformes, as suggested already^{8,9} though later challenged¹⁰.

The mandibular articulation of the *Gobipteryx* quadrate is atypical of the Palaeognathae, and resembles that of the pigeons: it is bicondylar without any structure corresponding to the posterior condyle. Such a quadrate is also clearly similar to that found in some smaller theropods, such as *Gallimimus*¹¹ or *Dromaeosaurus*¹², with the orbital process corresponding well to the pterygoid flange of the dinosaurs.

Palaeognathous birds are commonly thought to be of southern origin¹³⁻¹⁵, although this view was challenged for struthionids and aepyornithids¹⁶. Moreover, the dispersal of palaeognathous birds between the Old and New Worlds is explained only by the Gondwanian connections¹³. The Asiatic origin of *Gobipteryx*, the oldest known representative of the group, gives some evidence in favour of the northern dispersal. If migration across Beringia was possible for small mammals¹⁷, it would be the more so for birds although the flying ability of *Gobipteryx* remains a vexing problem.

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Rivalrous texture stereograms

THE two eyes receive slightly differing views of the world and it has been known since the invention of the stereoscope, 140 yr ago, that these slight differences are utilised by the visual system for stereopsis—the perception of the relative depths of objects on the basis of binocular disparity. The central theoretical problem in stereopsis is simply stated: how is disparity information extracted from the two retinal images? We examine here various possible answers to this question.

The most obvious possibility, and historically the first to be considered, is that the brain identifies objects in each eye's image, notes their different relative locations and computes stereopsis accordingly. But a monocular object recognition stage is emphatically not a requirement for stereopsis. This has been clearly demonstrated by the random-dot stereograms of Julesz¹. These stimuli show that stereopsis can be perceived even when the two eyes receive

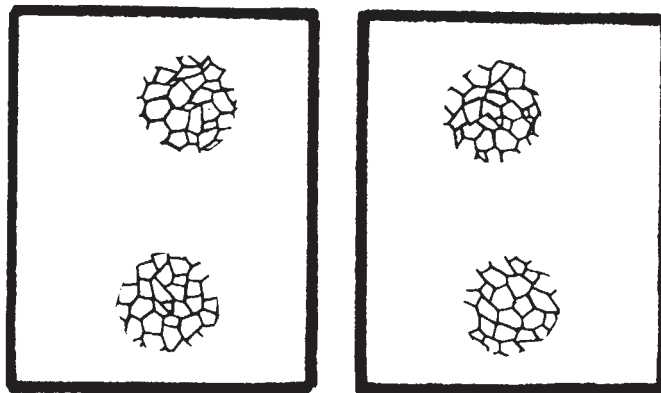


Fig. 1 Rivalrous texture stereogram (after Tausch²). Binocular fusion of the two halves of this figure results in the perception of relative depth (stereopsis) between two circular patches.

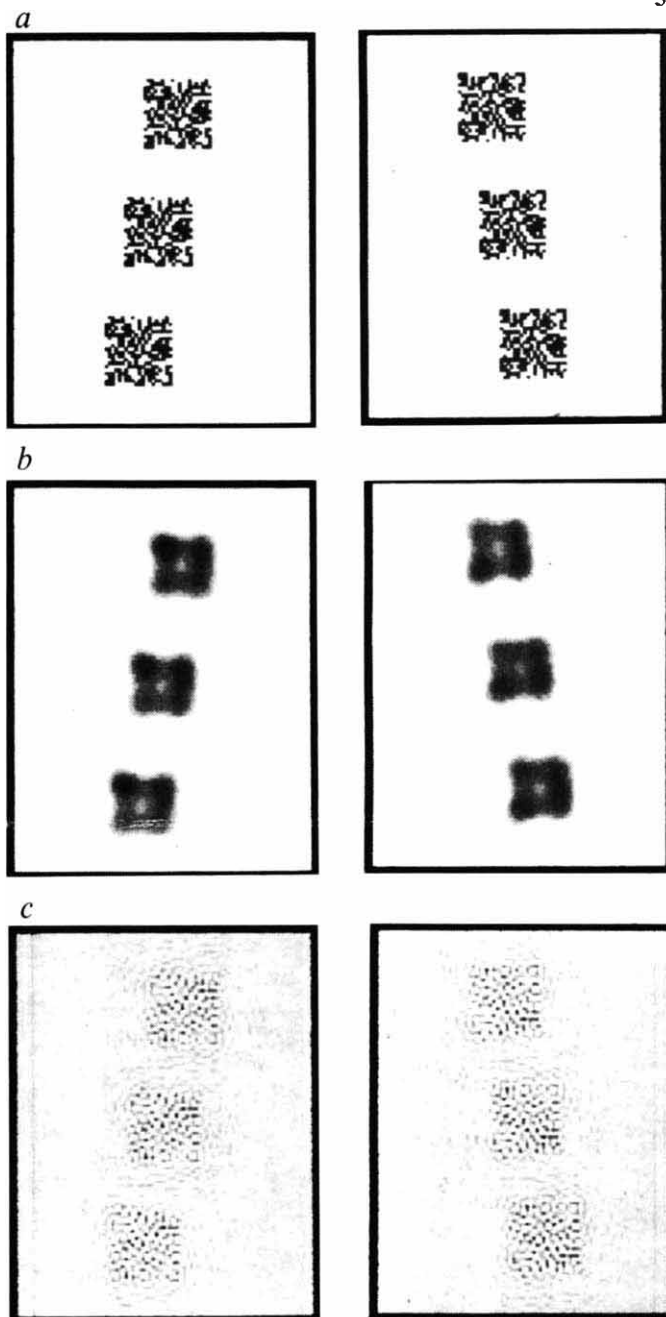


Fig. 2 *a*, Rivalrous texture stereogram in which three squares are seen to lie in different depth planes after binocular fusion although their textures are rivalrous. Each texture is an upside-down mirror image of the corresponding texture in the other field. This renders each pair of textures effectively random as far as binocular fusion is concerned, and hence rivalrous, and yet ensures that the left and right images have identical power spectra. The central squares have zero disparity with respect to the outer frame whereas the upper and lower squares have crossed and uncrossed disparities respectively. Presenting three squares in this way facilitates judgements of stereopsis, particularly in the relatively difficult circumstances of rivalrous texture stereograms, because if stereopsis is present not only do the squares appear in different depth planes but they also tend to appear vertically aligned. It is a methodological improvement over single-square figures³, in which it is not always easy to know whether the segregation of the square from its ground is presenting a masking depth cue which gives a depth impression independently of stereopsis. *b*, Low pass filtered version of (*a*) which contains only spatial frequencies below 1.0 cycle per degree if viewed from five times picture height. *c*, High pass filtered version of *a* containing only spatial frequencies above 4.0 cycles per degree. The original stereograms were prepared using a full-tone facsimile picture receiver monitored with a UDT photometer and viewed with back illumination. Photographic and printing processes inevitably introduce some spectral distortion from the originals but the various stereo effects which can be observed are faithful nonetheless.

random textures in which are embedded disparate forms completely hidden to monocular view but which nonetheless appear in depth due to the fact that their random textures are correlated in the two fields. This finding has led Julesz to conclude that there must exist stereopsis mechanisms which operate by effecting a 'low level' point-for-point matching of the two retinal images.

Given then that monocular object recognition is not a necessary precursor of stereopsis, it is interesting to ask whether it is a sufficient precondition. Consider Fig. 1, for example. This 'rivalrous texture stereogram'² successfully presents disparity depth cues by means of 'object' information even though there is only random correspondence between the left and right images of these objects at the level of fine texture elements. It is tempting to conclude from this demonstration that, as a 100% point-for-point matching of the left and right images of the objects is clearly impossible, a stereopsis mechanism sensitive simply to monocularly-discriminable shapes, irrespective of their precise detailed textures, must be at work and that therefore the monocular object recognition of disparate objects is indeed a sufficient basis for stereopsis³.

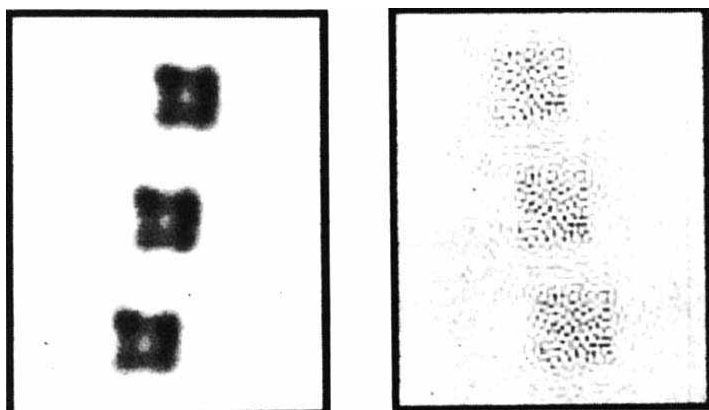
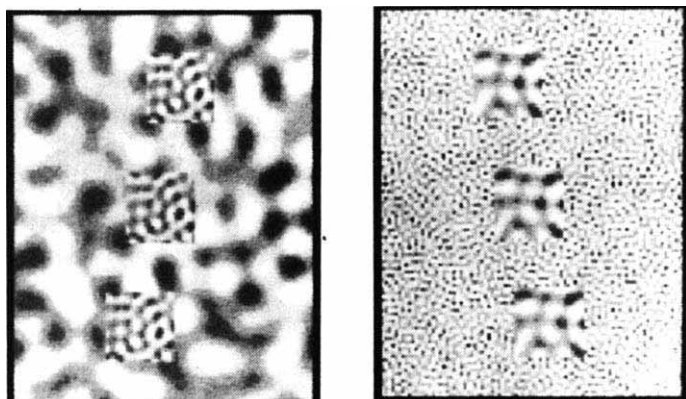


Fig. 3 Stereogram comprised of the left half of Fig. 2b and the right half of Fig. 2c. Stereopsis is impossible.

One conceivable way of accounting for the stereopsis of Fig. 1 in point-for-point terms, however, is to suppose that point-for-point matching follows a stage of spatial frequency filtering. Thus the disparity information carried by the objects might be extracted by point-for-point matching in a 'stereopsis channel' tuned to low spatial frequencies; while at the same time other stereopsis channels tuned to higher spatial frequencies, and thus sensitive to fine texture details, are in states of rivalry. This possibility is supported

Fig. 4 Stereogram comprised of random textures with no overlapping spectral content. Right hand: squares, 1.33 to 1.75 cycles per degree; surround, 4.0 to 5.25 cycles per degree. Left hand: squares, 2.3 to 3 cycles per degree, surround, less than 1 cycle per degree. Stereopsis is impossible.



by the demonstrations of Julesz and Miller⁴ which show that in random-dot stereograms depth can indeed be mediated by one spatial-frequency-tuned channel while others remain in rivalry.

We have tested this explanation using low and high bandpass filtered versions of the rivalrous texture stereogram shown in Fig. 2a. Figure 2b (low pass) and c (high pass) would selectively stimulate low and high spatial-frequency-tuned channels, respectively. As predicted, stereopsis is clearly present in the low bandpass filtered stimulus. Stereopsis, however, can also be established without difficulty in the high bandpass stereogram. This result shows that the idea of only low spatial-frequency-tuned channels being capable of mediating the depth seen in rivalrous texture stereograms is false: such channels would

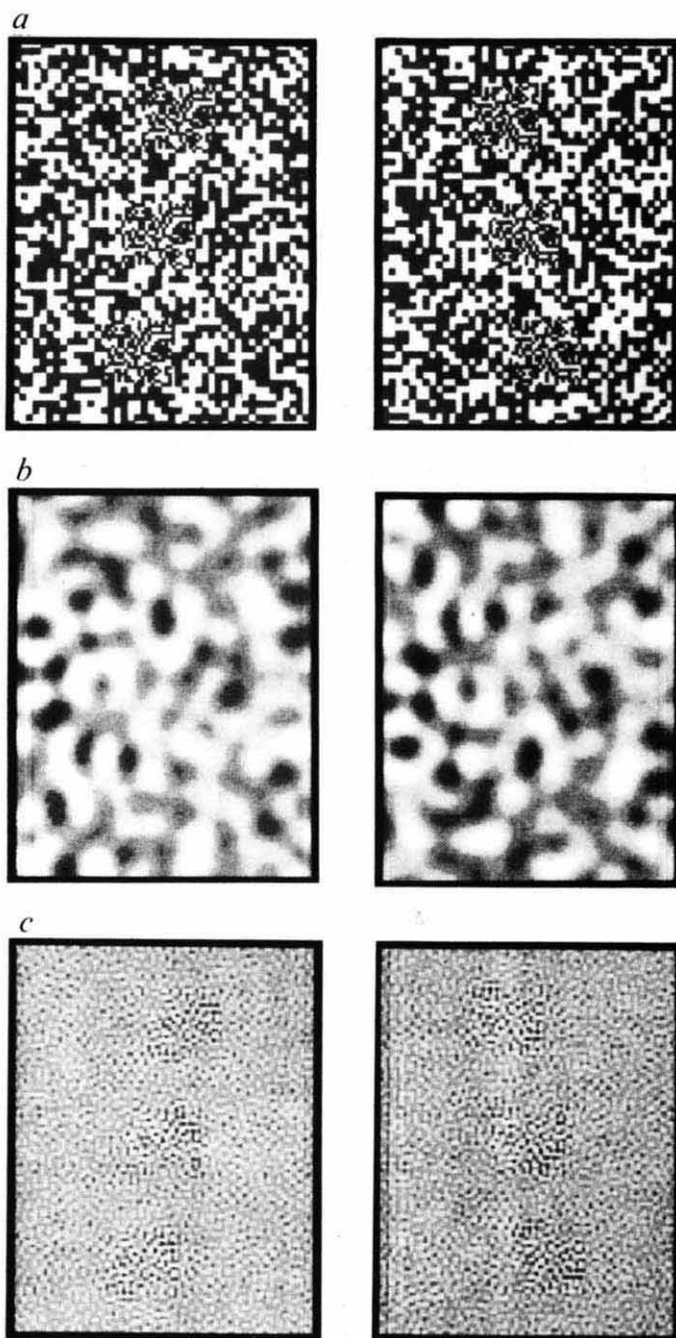


Fig. 5 a, Stereogram in which both squares and their surround are comprised of rivalrous textures (after Ramachandran *et al.*³). Rivalrous textures have been created as for Fig. 2. b, Low pass (1 cycle per degree) version of (a). c, High pass (4 cycles per degree) version of (a). Stereopsis is possible in (a) and (c) but with greater difficulty than in Fig. 2c.

not be active in the case of Fig. 2c and yet stereopsis is present.

The fact that stereopsis can be perceived in Fig. 2c suggests once again the operation of a 'monocular-object' stereopsis mechanism which is picking out the monocularly-visible texture boundaries of the squares and ignoring the fact that the details of the left and right textures do not match. It is therefore instructive to ask whether it is possible to obtain stereopsis by combining the left half of Fig. 2b with the right half of Fig. 2c. The squares in each of these half stereograms are of course perfectly visible monocularly and if there existed a mechanism capable of being driven by gestalts formed by texture contours³, then such a combination should surely produce stereopsis. Figure 3 represents this combination and we find stereopsis impossible. The two fields are extremely rivalrous. This result suggests that stereopsis can be obtained from rivalrous texture stereograms only if there is spectral overlap in the two halves of the stereogram, irrespective of the monocular visibility of the texture contours themselves. This conclusion is further supported by Fig. 4, which presents squares composed of rather less markedly different spatial frequencies in the two fields than Fig. 3 and sets them in surrounds also differing in spatial frequency. Again stereopsis is impossible, in spite of the existence of clearly discriminable and disparate monocular gestalts.

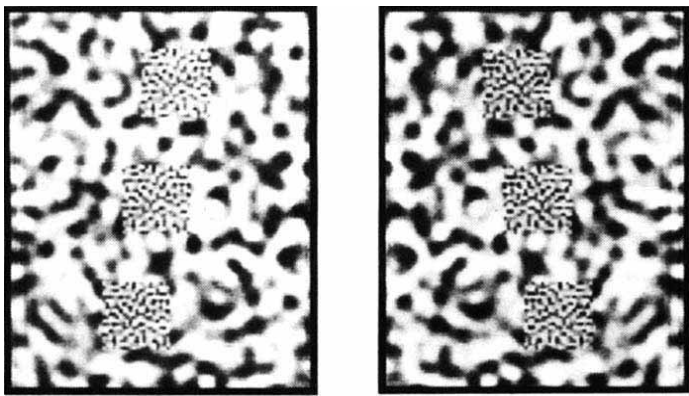


Fig. 6 Rivalrous texture stereogram in which the squares are comprised of spatial frequencies above 3.5 cycles per degree and in which the surround is comprised of spatial frequencies below 1.75 cycles per degree when viewed from five times picture height. Stereopsis is easy to obtain.

Figures 3 and 4 suggest that the notion of spatial-frequency-tuned stereopsis channels is valuable when considering rivalrous texture stereograms. But if such channels are to explain the stereopsis of Fig. 2c, and these channels are to be considered as involved in a process of point-for-point matching, then an account must be offered of how such channels could output stereopsis for Fig. 2c even though the textures of its squares have only random correspondence.

We submit that Fig. 2c has, for a high spatial frequency channel, what might be called partial point-for-point correspondence, rather than no correspondence whatsoever. First, note that the almost uniformly grey (d.c.) surrounds to the squares in Fig. 2c would not produce above-threshold activity in high spatial-frequency-tuned units. An array composed of such units would, quite simply, be 'blind' to these surround areas and not transmit forward any information about bright or dark points for point-for-point stereopsis processing. (The fact that our original high spatial frequency filtering produced these grey surrounds could be misleading here. If Fig. 2c is considered not as a stimulus but as a representation of activity in a high spatial-

frequency-tuned channel when stimulated by Fig. 2a, then it must be remembered that the grey level in this representation is the baseline or noise activity level, with above threshold departures from this level, bright or dark, being the only transmitted information to later stages of processing. That is, the white points in Fig. 2c can be regarded as reflecting above-threshold activity in high spatial-frequency-tuned neural units with on-centre/off-surround receptive field profiles, the black points as above-threshold activity in similar units with off-centre/on-surround profiles and the grey points as below-threshold activity in both these kinds of units.) Second, consider the possible matches which could be established for brightness points lying within the 'disparate' areas of the squares (that is, those areas 'confronted' with d.c. surround in the other field of view). These points could find no matches whatsoever at non-disparate locations, simply because the d.c. would not produce any above-threshold activity at these locations. If they are to find a match at all it would have to be a disparate one. And the possibility arises that a sufficiently large number of such matches occurs for stereopsis to be generated. Of course, there will never be a state of perfect point-for-point matching obtained for these areas, due to the inherently random selection of the two textures forming the squares. Nonetheless, a state of partial point-for-point correspondence does obtain and, as Julesz' (pages 271-275) has amply demonstrated, partial correspondence is an adequate basis for stereopsis. Moreover, there is no reason, in principle, why a point-for-point mechanism should require perfect correspondence as a *sine qua non* of its successful operation.

We report elsewhere³ a more detailed exposition of this idea and a development of Julesz's dipole model of stereopsis' which would be capable of providing the hypothesised binocular matching. At present we simply note some evidence which supports the idea in general terms.

Our explanation of the stereopsis of Fig. 2c depends crucially on there being no matches available in the surround areas. This leads naturally to the following question: what would happen if both the squares and their surrounds were composed of rivalrous textures? Such stereograms have already been devised³ and an example is shown in Fig. 5a. The squares of this stereogram are discriminable simply by virtue of the fact that their texture elements are half the size of those in the surround. Stereopsis from this stereogram is poor and unstable, as the inventors of this stereogram note and as the reader may judge. It is certainly of greatly reduced quality compared with the stereopsis from Fig. 2a-c. Figure 5b and c presents respectively low and high pass versions of Fig. 5a. As would be expected, the squares do not exist as monocularly-discriminable entities in either half of Fig. 5b. Fusion of this stereogram usually results in complete rivalry although we find that some observers occasionally report depth impressions relating to certain blobs which happen, through the random processes of manufacture, to have *ad hoc* and fortuitous disparities. In Fig. 5c the squares do exist as monocular entities although in a much attenuated form when compared with the squares in Fig. 2c. We find that stereopsis from this figure is possible although it is not obtainable as readily as in Fig. 2c, nor is it as stable. These impairments, caused by including a rivalrous surround, are exactly what would be expected given our general interpretation because local processes serving the squares in Fig. 5c can now obtain a better degree of matching with their surrounds than can the squares of Fig. 2c. Of course, given that the squares in Fig. 5c are still characterised by slight brightness differences from their surrounds, one would still predict that stereopsis should be possible because square/square matches will have a slight edge over square/surround matches. Nonetheless, because the brightness differences characterising the squares are not very marked, this superiority would not be as great as in Fig. 2c—hence the

fact that stereopsis can be obtained in Fig. 5c but in an impaired form.

One interesting prediction that can be drawn from our analysis of Fig. 5a is that stereopsis should be clear and easy to obtain from stereograms with rivalrous texture surrounds if the spectral content of the surround is sufficiently different from that of the squares. In such cases, the spatial frequency tuning of the channel dealing with the squares will result in this channel effectively dealing with a uniformly grey surround (as in Fig. 2b and c). Figure 6 presents a stereogram of this type and it produces clear stereopsis, as predicted.

The important conclusion deriving from our analysis is that stereopsis in rivalrous texture stereograms can be explained by spatial-frequency-tuned stereopsis channels without any need to involve more complex stereopsis mechanisms incorporating monocular object recognition.

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Role of stimulus environment and duration of processing in long term memory

A MAJOR concern of current memory research is to identify how information is encoded and transferred from short term memory (STM) to long term memory (LTM)^{1,2}. It has been reasoned that while an item remains in STM, it can be maintained through rehearsal; as a consequence, the corresponding LTM trace is built up. Thus, one may postulate that the adequacy of an item's registration in LTM is a positive function of the length of its stay in STM. Such a mechanical and simplistic account of memory has been challenged by a series of experiments which showed that when short term storage times were measured, these times did not predict long term retention^{3,4}. We report here an experiment which manipulated the processing time as well as the processing context and showed that the processing time had differential effects, sometimes beneficial, sometimes harmful, and sometimes neutral, on an item's long term retention, depending in which type of processing context the item was embedded. Such results argue for a perceiver-environment interactive theory of memory.

To minimise the difference between subjects' mnemonic strategies, the present experiment used an incidental-learning paradigm—that is, the subjects were not informed about the recall test until the very end of the experiment. Instead, they were told that the experimental task was a target-search task and were made to believe that our interests were the accuracy and the speed of their search. In fact, the experimental task was similar to the visual search task used by Neisser and Beller⁵ except for the final unexpected free recall test. In general, the subject was required to hold a target word in his memory and then to scan through a search list until the particular target word was found. By manipulating the target position within the search list and the nature of others in the list, we were able to vary the processing duration as well as the stimulus environment. In the present study, there were four different list structures and three different target positions, forming

a 4×3 factorial design with both variables as within-subjects measures.

The four list structures were made up in the following ways. To illustrate, let the target word be TABLE. For the phonemic-similar (PS) condition, all other list words were phonemically similar to the target—for example, STABLE, CABLE, MAPLE. For the semantic-similar (SS) condition, all other list words belong to the same semantic category as the target word—for example, CHAIR, SOFA. For the random (RAN) condition, all other list words were randomly selected from the Thorndike-Lorge's high frequency words. Finally, for the nonsense (NON) condition, all other list items were nonsense but pronounceable letter strings with length ranging from three to five. The target words were always embedded in list position 3, 9 or 15. The search lists were always 20 items typed (double spaced) vertically in a column. The subjects were 40 undergraduate students enrolled in an introductory psychology course at the University of California (Riverside), and were tested individually.

The subject was instructed to search the list from the top to the bottom, using a pencil to direct his attention to each word. The subject was instructed to search for the target word as quickly and as accurately as possible, and to place a check beside the target word as soon as it was found. The experimenter recorded the search time for every word with a stop watch. Two practice trials with numbers preceded the experimental task of 12 target searching trials. After the last searching trial, the subject was asked to fill in a questionnaire which included some autobiographical data—for example, name, address, phone number, and so on, for about 2 min. An unexpected recall test was then administered. The subject was asked to write down as many target words as he could in any order he preferred. This recall interval was fixed at 2 min. A post-experimental interview revealed that no subject had anticipated such a "tricky" test.

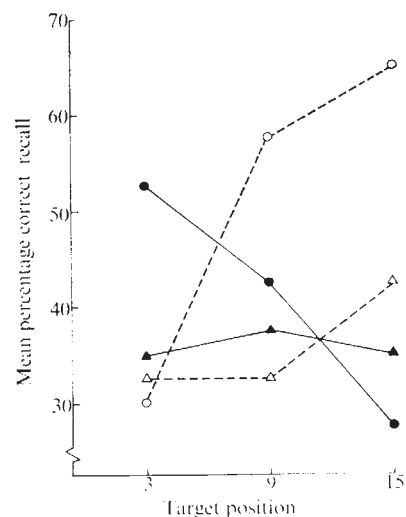


Fig. 1 The subject was asked to hold a target word in his memory and then to scan through a list of words until that particular target word was found. At the end of 12 such visual search trials with 12 different target words, an unexpected recall test for all the target words was administered. Mean percentage recall of the target words was plotted in this figure as a function of the processing time and the processing context. The length of the processing time was manipulated by placing the target word at one of the three positions (that is, 3, 9 and 15) within the search list. The processing context was manipulated by varying the nature of the search list: ●, all other words in the search list were phonemically similar (PS) to the target word; ▲, all other words in the search list were semantically similar (SS) to the target word; △, all other words in the search list had no apparent relation to the target word (RAN); ○, all other items in the search list were non-words (NON) made up with nonsense syllables.

The results of the experiment are summarised in Fig. 1. Mean percentages of correct recall in various list conditions were plotted as a function of the target position. It is clear that the duration of processing has differential effects on the item's long term retention, depending on the stimulus environment in the processor at the time of processing that item. Statistical tests (ANOVA) substantiated the interaction between target position and list condition ($F(6, 234)=4.78, P<0.01$).

The first striking aspect of the data depicted in Fig. 1 is the fact that when the target was embedded at position 3, the PS list condition produced the best recall performance compared with the other three conditions (all P values <0.05 with Tukey's test) which did not differ from one another. This is a very intriguing finding. Most current memory theories would predict that since the item would be better retained in a non-interfering environment than in an interfering context, recall performance would be worst in the PS condition where the processor is filled with acoustically similar items. But the data showed that this prediction holds only at position 15. The relative superiority of the PS condition at positions 3 and 9 is difficult to account for by current memory theories.

From the viewpoint of a perceiver-environment interactive model, however, the superiority of the PS condition at position 3, followed by a gradual decline at position 9 and 15, can be readily explained. According to Neisser and Beller⁵, in a search task the subject needs only to hold a phonemic representation of the target word while he is searching the list. The immediate match between the temporarily held representation and the acoustically similar environment pushes the recall level up about 20%. This facilitating effect of a phonemically similar environment suggests that an acoustically similar sound helps to confirm and establish a retrieval cue which is phonemic in nature. At position 3, the newly established phonemic cue is relatively unique and distinct. As there are more acoustically similar items to be searched, however, this memory code becomes less unique and less distinct due either to storage deterioration (an 'acid-bath' hypothesis)⁶ or to a retrieval confusion (a 'cue-overloading' effect)⁷. Thus, in the PS condition, short processing (but long enough to have three or four items processed) has a positive effect on the item's retention. Once the phonemic code is established, however, the longer the processing time, the more items will be checked into the processor. Subsequently, prolonging the processing time has a negative effect on item's retention.

When the target word was embedded in an environment which bears no apparent relationship to the word itself, such as in the RAN and the NON conditions, the duration had no effect on the item's retention. For no matter how long the item has been held in the processor, there will be little, if any, possibility of interaction between the item and its environment.

When the item was embedded in a semantically related context, the results showed quite a different pattern. The phonemically encoded representation of the target word did not match the environmental context. In fact, the environment contained information related to the semantic rather than the phonemic aspect of the target word. The results of position 3 suggest that only two semantically related items are not enough to activate a semantic code for the target item. Thus, the SS condition functioned like the RAN and NON conditions in that recall was not facilitated by the environmental context. The results at positions 9 and 15 suggest that when the number of semantically related words searched is large enough to activate a semantic code, the recall probability is suddenly boosted and continues to grow from position 9 to 15. Thus, when the initial representation does not match its environment which contains some useful information by itself, increasing the processing time increases the possibility of discovering

that information. In these conditions, duration of processing does have a positive effect on the item's retention.

The pattern of results from the present study suggests that the effect of processing duration on item retention is a very complicated matter. An item is not processed in a vacuum; rather, the processor is always shared by other on-going activities which become a functional environment for the processed item. To the extent that the environment does contain some information which will help the processor to establish a more durable memory code, processing time will be beneficial. When the environment contains no systematic information to help the processor to establish a unique and distinct code, time itself is not an important factor.

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Relationship between reward-enhancing and stereotypical effects of psychomotor stimulant drugs

BIEULER has described stereotyped behaviour as "one of the most striking external manifestations of schizophrenia"¹. Schizophrenic stereotyped behaviour has been found in the spheres of movement, action, posture, speech, writing, thought and desire¹. Abuse of the psychomotor stimulant drugs, such as the amphetamines, methylphenidate, cocaine and pipradrol, can cause a psychosis which is clinically indistinguishable from paranoid schizophrenia², and which contains stereotyped components^{2,3}. In animals, acute doses of the stimulants can induce stereotyped behaviour which is apparently under minimal situational control, and which can disrupt normal activity⁴. In contrast, low doses of the stimulants can apparently improve learning and performance in a variety of situations, in both animals⁵ and man⁶. This paper demonstrates a relationship between these two apparently diverse actions.

The facilitation of behaviour by stimulants has been attributed to the enhancement of the effects of rewarding stimuli, by the potentiation of a brain reinforcement mechanism⁷. One of the major functions of reinforcement is to effect learning, an associative process, and it is possible for neutral stimuli, which are subsequently paired with primary rewards, such as food or water, to acquire reinforcing properties. These stimuli are termed conditioned reinforcers (CRs), and there is some evidence that stimulants can augment the effects of conditioned reinforcement^{8,9}, thus supporting the reward-enhancement hypothesis. It is, however, unclear how this hypothesis, or any other that has been advanced to explain the behavioural effects of stimulants^{10,11}, can account for the stereotyped behaviours produced by these drugs. This paper will show that stimuli with acquired motivational significance (CRs) can be crucial determinants of the selection of responses which form part of a stereotyped pattern of behaviour. Experiment 1 shows

that a dose of 10 mg kg^{-1} of the stimulant drug pipradrol, can facilitate the learning of a novel spatial discrimination task reinforced solely by a stimulus formerly correlated with reward. Experiment 2 demonstrates that although CR stimuli are important in the selection of the responses which are stimulated by pipradrol, these responses are performed in a perseverative manner, being part of a stereotyped pattern of behaviour induced by the drug.

Experiment 1 consisted of two stages. In stage 1, rats were trained either to associate, or not to associate, a specific stimulus with the presentation of water. Stage 2 tested the strength of the association formed in training, by using the stimulus as a reinforcer to sustain new learning, a stringent criterion for the establishment of conditioned reinforcement¹². Male albino rats (150–160 g at commencement of training) were provided with food *ad libitum* and subjected to a 23-h water deprivation schedule throughout the experiment. Initially, they were trained to push a panel in a standard Skinner box to collect water from an elevated dipper⁹. During stage 1, the dipper operated for 7.5-s periods, at intervals of 30 s for a total of 30 reinforcements per session, for two sessions. On three further sessions, water rewards were programmed to occur at variable intervals, averaging 30 s.

There were two conditions, CR and FS, which used different groups of rats. In the CR condition ($n=14$), a light preceded each presentation of water by 0.5 s, remaining switched on for the duration of the reinforcement. It was thus perfectly correlated with reward and was the CR stimulus. In the FS condition ($n=14$), the same light was presented according to a similar schedule, but it was randomly correlated with reward. It therefore had no associative relationship with reward and was merely a familiar stimulus (FS). There were two levers in the box during training, situated to the left or the right of, and equidistant from, the panel. Responding on either lever had no consequence for the animal in stage 1.

For stage 2, there were three 30-min sessions, each separated by 48 h to allow recovery from drug effects. No rewards of water were given during these three sessions. Animals were divided into equal drug and control groups (both $n=7$) within the CR and FS conditions. The drug groups received intraperitoneal injections of 10 mg kg^{-1} pipradrol, 15 min before each of the 30-min sessions. This dose was used since it has been found to be effective for studying the potentiation of CR by the drug in another paradigm⁹. In addition, the purpose of this experiment was to manipulate behavioural variables, while keeping pharmacological ones fixed. The control groups received injections of the drug vehicle (1:2 mixture of polyethylene glycol and distilled water). In both conditions, CR and FS, pressing one of the two levers in the box now produced the light stimulus with $P=0.5$, whereas responding on the other lever had no programmed consequence. The lever providing the stimulus was counterbalanced over subjects. This constituted a novel spatial discrimination task, reinforced solely by the light stimulus. Levers were designated as CR or NCR (no CR) or alternately as FS or NFS (no FS), in the two conditions. Responses on each lever, panel pushes and light presentations were totalled and recorded at 15 min intervals during the session.

The results obtained strongly support the hypothesis that the stimulation of responding by pipradrol was controlled by the CR stimulus. An analysis of variance was carried out on the lever press totals for each rat, in the drug and control groups of the CR and FS conditions. All data were subjected to a square-root transformation to preserve homogeneity of variance¹³. The analysis revealed a significant interaction of drug with condition with the lever ($F=9.10$, d.f.=1,24; $P<0.01$), which represents the highly specific action of pipradrol illustrated in Fig. 1. Responding was greatly elevated on the CR lever in the pipradrol group,

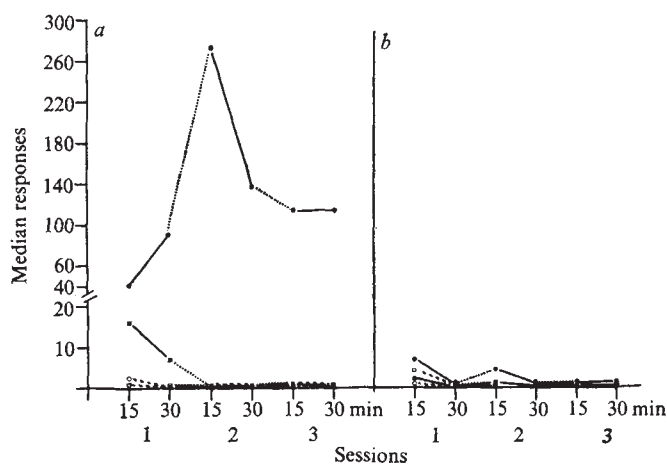


Fig. 1 Experiment 1: Median response totals in the CR (a) and FS (b) conditions for drug and control groups, for different levers, plotted at 15- and 30-min stages for each of the three 30-min sessions in stage 2. The use of medians to express these data is preferred, because it provides a more accurate description of the group data. The use of means would greatly inflate the scores for the drug group in the CR condition due to the contributions by certain rats, of very high response totals on the CR lever. The dotted lines between sessions indicate that these are not continuous functions of time. Note the change of scale on the vertical axis, to accommodate the low response totals of the control conditions. a: Pipradrol 10 mg kg^{-1} ; ●, CR; ■, NCR; Control: ○, CR; □, NFS. b: Pipradrol 10 mg kg^{-1} ; ●, FS; ■, NFS; Control: ○, FS; □, NFS.

although responding on the NCR lever quickly reached control levels and lower. This shows that the stimulant action of pipradrol was restricted to the CR lever and could not therefore have been due to nonspecific increases in responding. Further, the much lower level of responding in the FS condition, for the FS stimulus, shows that the CR condition results are not simply due to the drug enhancing the reinforcing properties of stimulus change¹⁴, but depend on the learned association of the stimulus with primary reward. Pipradrol also apparently enhanced the learning of the spatial discrimination task to obtain CR. Responding for the CR initially increased over time under pipradrol, whereas responding in all other conditions generally showed monotonic decrements. In fact, all seven rats in the pipradrol group responded more for CR on session 2 than on session 1. In addition, five of these seven rats showed increases in responding on the CR lever which were concomitant with decreases in responding on the NCR lever. This pattern is consistent with the interpretation that pipradrol facilitated the learning of the task to obtain CR, and that extinction of responding only began to occur later, as the association between the CR and water became weaker. The experiment emphasises the importance of stimuli of prior motivational significance in determining the selection of responses which are stimulated by pipradrol. It also provides the most convincing evidence to date that stimulant drugs enhance the effects of conditioned reinforcement, especially since the potency of CR to sustain new learning in control organisms is not great^{12,15}, a view supported by the control data of the present experiment.

Observations of the drug-treated rats using closed circuit television revealed that these animals displayed stereotyped patterns of behaviour, which could include repetitive sniffing, head movements, rearing in the vicinity of the CR lever, and more rarely, incipient approach responses to the panel. The important question then arises: are the lever presses, in the patterns of behaviour, goal directed, occurring in spite of the competing stereotyped activity, or do they represent the perseveration of responses that were once goal directed and now form part of a stereotyped pattern of behaviour? The second possibility suggests that pipradrol

exaggerated the differences in responding on the CR and NCR levers in experiment 1, by increasing the tendency to repeat responses. It also suggests that stereotyped behaviour can have learned components.

Experiment 2 tests between these hypotheses by using a task in stage 2 in which perseveration of responding acts against, rather than in favour of, learning. If it is possible to demonstrate that the lever pressing for CR has perseverative properties, then its similarity to other forms of stereotyped activity would be underlined. Stage 1 was similar to that of experiment 1, except that the CR stimulus was 0.5 s long. In stage 2, the CR consisted of this stimulus and the brief (0.3 s) elevation of the empty water dipper. To obtain CR, the learning of a two-component response sequence was required, involving a single response on each lever, made in the precise sequence, component 1, component 2. Thus perseveration of responding on either lever would lead to disruption of learning and a reduction in obtained CRs.

Rats trained as previously were injected with either 10 mg kg⁻¹ pipradrol ($n=6$), or vehicle ($n=6$), 15 min before each of six 30-min sessions. Figure 2 shows that there was a remarkably similar pattern of responding to that shown in experiment 1. There were sharp increases in responding on the second component of the response sequence, concomitant with decreases in responding on the first component, for drug-treated rats. This perseveration of responding led to a progressive reduction in obtained CRs, although the absolute response rate was still increasing.

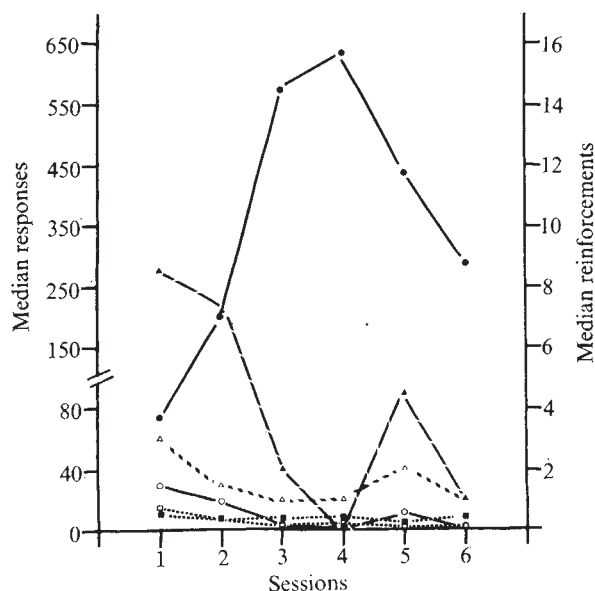


Fig. 2 Experiment 2: Median response totals plotted over the six 30-min sessions of stage 2 for component 1 and component 2 lever press totals. The differential stimulation of component 2 over component 1 in the drug group, as compared with the control group, is significant, following an analysis of variance similar to that used in experiment 1 ($F=6.43$, d.f. = 1,10, $P < 0.05$). Note the change of scale on the left-hand side vertical axis. Median CR totals are also plotted on the right-hand side vertical axis, as a function of sessions. Pipradrol 10 mg kg⁻¹: ●, component 2; ○, component 1; ▲, reinforcement; control: ■, component 2; □, component 1; △, reinforcement.

There were, in fact, no overall differences in the number of CRs obtained by the drug and control groups (Mann-Whitney U test, $P=0.132$, two-tailed). It is of great importance that the stimulation of responding in the pipradrol group always occurred for the component of the sequence closest in time to the presentation of CR. Although the selection of the response was once again controlled by the CR contingency, however, its performance was perseverative, since it resulted in a reduction of obtained CRs.

Pipradrol thus clearly failed to sustain the learning of the response sequence to obtain CR. The fact that responding was perseverative, and integrated into a stereotyped pattern of behaviour, as in experiment 1, shows, however, that it is probable that stereotyped behaviour can have learned components. It is of course difficult to say whether most forms of stereotypy result from the perseveration of responses formerly goal directed. It is clear that the learned components in the present patterns were not performed indefinitely; extinction of them occurred in most instances, showing that perseveration does not completely override environmental controls. It is interesting, however, that responding was very persistent in certain cases in experiment 2, in which the frequency of CR was low, therefore diminishing the opportunity for extinction of the original association of the CR with water. One rat showed increasing response rates over the six sessions, making more than 3,400 responses on the last session, receiving only one CR. The results from both experiments support the hypothesis that a general action of stimulant drugs is to cause increased repetition of responding, with response selection being dependent in part on environmental contingencies³. Thus the 'reward-enhancing' and stereotyped effects of the psychomotor stimulants are postulated to arise from the action of a common mechanism.

This hypothesis can explain the apparent conditioning of stereotypy in amphetamine-treated cats, as well as the subsequent 'fragmentation' and ritualisation of their responses into stereotyped patterns¹⁶. It can also explain the idiosyncratic nature of the stereotypies of human amphetamine addicts, which involve perseveration of well-established habits such as car maintenance and house cleaning³. Finally, it has implications for the apparently meaningless stereotyped activities of certain psychotic states, such as autism¹⁷ and schizophrenia^{1,2,18}. It is suggested that these activities may arise from a fixation on behaviour which had been initially purposeful. In support of this, Bleuler¹ has described schizophrenic stereotypies which were related to events of significance occurring in the previous history of the subjects. This paper has shown that an analogous mode of behaviour may occur in rats treated with amphetamine-like drugs, and strengthens the use of drug-induced behaviour as model psychoses.

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Influence of ethylene and oxygen on respiration and peroxide formation in potato tubers

APPLICATION of ethylene to potato tubers induces a respiratory upsurge¹, comparable with that in ripening climacteric fruit², but which can be studied independently of other ethylene-induced processes occurring in fruit. It has been suggested that ethylene catalyses oxygen-requiring processes, reflecting in part oxygen utilisation in the formation of peroxides³. We report here that high O₂ tensions, in combination with ethylene, markedly enhance the respiratory rise, which is accompanied by a corresponding increase in peroxides.

Locally grown potato tubers (*Solanum tuberosum* L., variety Northchip) were preconditioned at room temperature for 2 weeks after harvest. Four tubers weighing together approximately 1.0 kg were each placed in a 4-l jar and ventilated continuously, at a flow rate of 400 ml min⁻¹, with 21% (air) or 100% O₂, supplemented with 0 or 10 p.p.m. ethylene as previously described³. The potatoes were kept at 21 °C throughout the experiment. Evolution of CO₂⁴ and peroxide⁵ was monitored at 6-h intervals for 36 h. All determinations were run in duplicate.

Figure 1a and b shows that in the absence of ethylene respiration was at a steady rate in both air and 100% O₂, although in oxygen the rate was somewhat higher. Addition of ethylene to air resulted, after a lag, in a respiratory rise, reaching a peak two or three times the initial rate after

24 h, followed by a decline. By comparison, after a comparable lag, ethylene in 100% O₂ induced a rapid rise in respiration to almost ten times the initial rate.

The results show that although ethylene was required to trigger the respiratory rise, as previously observed¹, oxygen tension was the rate limiting factor. A similar interaction between oxygen and ethylene has been obtained in the induction of synthesis of lycopene, the red tomato pigment, in the non-ripening *rin* mutant³. As in potato respiration, ethylene was obligatory for the initiation of lycopene synthesis but O₂ tension was the rate limiting factor.

We hypothesised that the changes in the oxygen tension reflect, in part, peroxide formation and that ethylene triggers the process, because (1) ethylene stimulated peroxide formation in fruit⁶, and (2) peroxide forming enzymes are stimulated by high O₂ tensions⁶.

To test this hypothesis, we measured the changes in peroxides in the tubers as related to the effect of different O₂ tensions with and without ethylene. As with CO₂ evolution in the absence of ethylene, the peroxide level did not change in air or 100% O₂ (Fig. 1c and d, respectively). Addition of ethylene resulted in an upsurge in the formation of peroxides. As with CO₂ evolution, the peroxide levels were stimulated by high O₂ tensions.

Solomos and Laties⁷ proposed that ethylene triggers an alternative respiratory pathway (cyanide insensitive). Our demonstration that the respiratory rise, as influenced by ethylene and oxygen, is accompanied by an upsurge in peroxides suggests that the alternative respiratory pathway leads to the formation of peroxides.

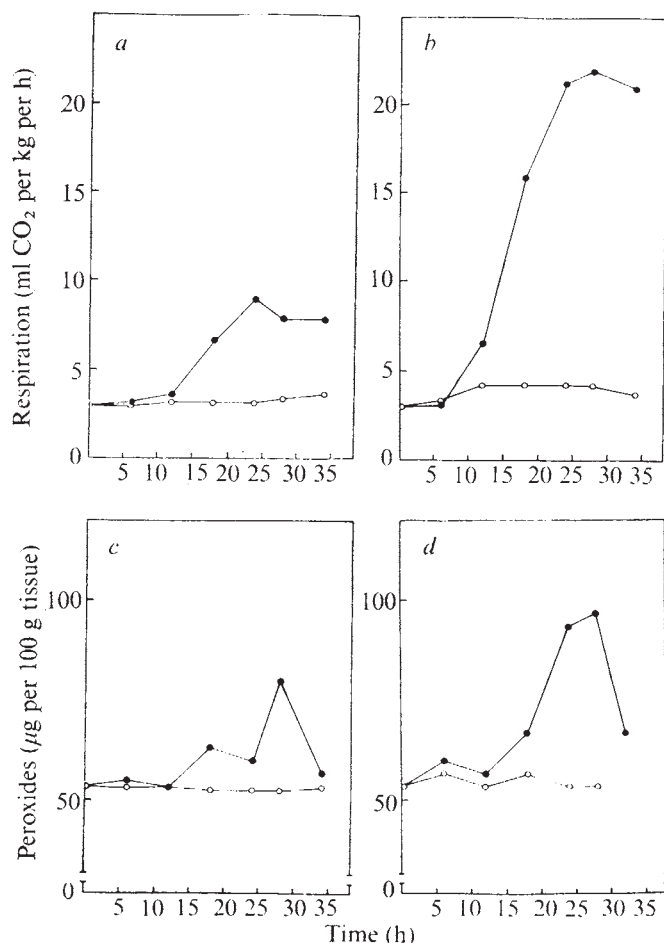
The formation of peroxides in plants is catalysed by several enzyme systems⁸, but may also result from the dismutation of superoxides⁹. In any case, the upsurge in peroxides represents the formation of partially reduced oxygen forms, while in respiration molecular oxygen is reduced completely (formation of water). The action of ethylene, as shown in potatoes, may therefore be to divert the flow of electrons from the complete reduction of molecular oxygen to the formation of partially reduced oxygen forms, such as peroxides, which by comparison with O₂, represent oxygen forms which readily react with cellular constituents^{8,9}. In this way, ethylene may induce redox changes in tissues, including the oxidative breakdown of senescence-retarding hormones in fruits⁹, and thereby the onset of senescence processes.

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Fig. 1 Effect of ethylene on CO₂ evolution (a and b) and peroxide formation (c and d) in air (21% O₂) and 100% O₂. Ethylene concentrations were zero (○) and 10 p.p.m. (●).



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Characterisation of human cell lines and differentiation from HeLa by enzyme typing

THE value of a great deal of research on cells in culture depends on the certain identity of the cells under investigation. Contamination of one cell line with another, leading to mixed cultures or in some cases complete overgrowth of the original cells by the contaminating line, is a long-standing problem. Interspecific contamination has been

recognised by both immunological and karyological techniques^{1,2}, but the most striking demonstration of intra-specific contamination was by Gartler³. He presented evidence, based on the detection of common genetically determined variation in two enzymes, that many permanent tumour cell lines, set up originally in several different laboratories, were in fact HeLa cells. Recently the problem of contamination with HeLa has become a focus of general interest and there has been a search for certain chromosomal and other characteristics of HeLa in a large number of established lines⁴⁻⁶. There is no guarantee, however, that the contaminating cell line will always be HeLa and there appears to be a need for a quick and reliable method for the absolute identification of all human cell lines.

In recent years more has become known about genetic diversity in man, and many electrophoretically distinguishable enzyme polymorphisms have been discovered⁷. Most of this information has come from studies on red cells and placentae, but many polymorphic loci are expressed in cultured cells. Thus it is already possible to devise a relatively simple scheme which goes a considerable way towards the positive identification of individual human cell lines. As new polymorphisms are discovered it will doubtless be improved. We present here our current list of enzyme polymorphisms which we have found useful for the identification of all types of cultured cells, including diploid and transformed fibroblasts and permanent lymphoblastoid lines. We have been able to test out the scheme

Table 1 Enzyme loci useful in characterising human cultured cells

Enzyme		Population group*				Refs
Locus	Phenotype	N. European Frequency of common phenotypes	Chance of 2 people being alike	Black Frequency of common phenotypes	Chance of 2 people being alike	
PGM_1	1	0.57		0.62		7
	2-1	0.37	0.465	0.33	0.496	
PGM_2	2	0.06		0.05		8
	2-1	1.00	1.000	0.98	0.961	
PGM_3	2	—		<0.01		7+
	1	0.55	0.452	0.14	0.392	
GOT_M	2-1	0.38		0.47		7+
	2	0.07	0.942	0.39	0.739	
ESD	1	0.97		0.85		7
	2-1	0.03	0.701	0.02	0.701	
AK_1	3-1	—		0.13		7
	Others	<0.01	0.853	<0.01	1.000	
ADA	1	0.82		0.82		7
	2-1	0.17	0.820	0.17	0.887	
ACP_1	2	0.01		0.01		8
	1	0.92	0.321	1.00	0.555	
PGD	2-1	0.08		—		9
	2	<0.01	0.923	0.94	0.786	
$G6PD$	1	0.90		0.06		10‡
	2-1	0.10	1.000	<0.01	0.507	
$PEP A$	2	<0.01		<0.01		7§
	A	0.13	0.905	0.03	?	
$PEP C$	BA	0.42		0.28		11*
	B	0.35	0.961	0.69	?	
$ACON_s$	CA	0.04		<0.01		7+
	CB	0.06		<0.01		
$PEP A$	Others	<0.01		<0.01		7+
	A	0.96	0.923	0.88	0.786	
$PEP C$	AC	0.04		0.11		10‡
	Others	<0.01	1.000	<0.01	0.507	
$ACON_s$	B	1.00		0.56		7§
	A	—	1.000	0.44	0.689	
$PEP A$	1	1.00		0.81		11*
	2-1	—	1.000	0.18	?	
$PEP C$	2	—		0.01		7+
	1	0.95	0.905	?	?	
$ACON_s$	4-1	0.05		?		7+
	Others	<0.01		?		
$PEP A$	1	0.98		0.73		7+
	2-1	<0.01	0.961	0.05	0.575	
$PEP C$	3-1	0.01		—		7+
	4-1	—		0.20		
$ACON_s$	4	—		0.01		7+
	Others	<0.01		0.01		
Combined chance of two people being alike for all loci			0.025		0.008	

PGM_1 , PGM_2 , PGM_3 : first, second and third loci of phosphoglucumutase. GOT_M : mitochondrial glutamate-oxaloacetate transaminase. ESD : esterase D. AK_1 : adenylate kinase. ADA : adenosine deaminase. ACP_1 : 'red cell' acid phosphatase. PGD : phosphoglucuronate dehydrogenase. $G6PD$: glucose-6-phosphate dehydrogenase. $PEP A$ and $PEP C$: peptidases A and C. $ACON_s$: soluble aconitase. Enzymes bracketed together were routinely run on the same gel. Electrophoresis and detection of enzymes were by methods previously described⁷.

*The phenotype frequencies of many of the enzymes show marked regional variations even within the same ethnic group. The most reliable data are from the North European population surveys. Wherever possible the phenotype frequencies given for the Black population are derived from population studies on Yorubas from Nigeria.

‡Enzyme not found in red cells.

§The $G6PD$ A phenotype includes both $A(-)$ and $A(+)$, since these are difficult to distinguish in cultured cells. The frequency of the A and B phenotypes is very variable in different populations and this estimate is from Nigerian males. We have used the male frequencies to calculate the chance of two cell lines being alike since a line in long-term culture might be expected to be effectively monoclonal and thus to express only 1 allele at an X-linked locus such as $G6PD$.

¶The frequencies given for $PEP A$ ignore the $PEP A^+$ allele which is technically difficult to distinguish from $PEP A^1$.

*Some alleles at the $PEP C$ locus are not expressed in red cells and reliable population data are not available for the Black population (see refs 7 and 11).

on five permanent epithelial cell lines derived from human bladder tumours. These results are presented together with findings on nine other permanent human cell lines, all of which seem to be, or are known to be, HeLa.

Details of the enzymes used in our cell identification scheme are given in Table 1. The loci selected are in general those which have useful gene frequencies in different populations, and which have been studied in detail in both cultured cells and in families. All the loci are expressed in other tissues, and ten of them are expressed in red cells (Table 1) so that the best positive check on the identity of a cell line can be made if blood or other tissue is set aside for enzyme testing when the cells are put into culture. Using starch gel electrophoresis it is not necessary to run a separate gel for each enzyme. As Table 1 shows, the seven enzymes providing the most information (the first seven tested, excluding PGM₂) are usually run on only three gels, and could be done on fewer if necessary. Using routine methods the enzymes suggested could all be done on a single pellet of 10⁷ cells.

Three of the enzymes listed in Table 1 require further comment. Adenosine deaminase (ADA) and occasionally acid phosphatase (ACP₁) may be difficult to type in diploid fibroblasts, because of a preponderance of 'tissue' isozymes in which genetic variation is not detectable. The adenylate kinase locus (AK₁) is not expressed in lymphoid lines. In our experience all the loci listed in Table 1 are readily typeable in permanent epithelial lines.

Table 1 also shows the chance of being able to distinguish any two individuals, of similar racial origin, using these enzyme loci; this turns out to be about 97% in North Europeans and about 99% in Blacks. The chance of distinguishing any two lines of dissimilar racial origin would of course be greater. Further information may be obtained by testing other enzymes which have been omitted from this scheme because variants occur only at low frequencies. Some cell lines would be found to be heterozygous for one of these variants if sufficient loci were tested¹². There are also several fairly recently discovered enzyme polymorphisms that have not yet become established in our laboratory as routine markers for cultured cells, but which will probably be useful in the future. These include mitochondrial malic enzyme^{13,14}, glyoxalase I¹⁵ and uridine monophosphate kinase¹⁶. α -Fucosidase may also be included in the scheme, but it requires an additional technique, isoelectric focusing^{17,18}.

The results of testing a series of human cell lines by the scheme outlined above are given in Table 2. Five lines were derived from bladder tumours and maintained at the

Imperial Cancer Research Fund Laboratories. All these were of North European origin: RT4 was an English male¹⁹; RT112 an English female (C. C. Rigby and L.M.F., unpublished observations); T24 was a Swedish female²⁰; J82 was a Swedish male (provided by Carol O'Toole, University of California) and EJ was an American male (provided by J. Daly, Massachusetts General Hospital). Most of the other lines are well known strains of HeLa and all except BU25 (ref. 21) have featured in a recent investigation of cell identity⁵. The two remaining lines, LU and EUE, were originally set up as independent lines from foetal lung and subcutaneous foetal tissue respectively^{22,23}. They were sent to us for typing because they were suspected of having been taken over by HeLa and in fact the enzyme types were found to be those characteristic of HeLa cell lines. All the cells tested were sent to the Galton Laboratory in the form of washed cell pellets.

Clearly the bladder tumour lines are not the same as HeLa and differ among themselves. Three of them have unique enzyme phenotypes. The other two, J82 and EJ, are the same as each other on the enzymes tested in Table 1 and so appear to be identical. This is unfortunate but statistically not too distressing since if the chance of any two individuals being the same is 3%, the chance of finding two alike in a sample of five lines is about 1 in 4. Thus the evidence at present is not strong enough to suggest that one line has been taken over by the other, but clearly this is a possibility. Attempts to distinguish the two lines using other enzymes including α -fucosidase and glyoxalase I have so far been unsuccessful. They both seem to be phenotype α FUC 2-1 and GLO 1. The chance of two lines from different individuals being identical for these two enzymes is about 1 in 4. The two lines do, however, differ in other properties (C. J. Marshall, L.M.F. and A. W. Carbonell, in preparation). EJ cells produce tumours in nude mice and have a colony-forming efficiency in agar of 23%. J82 cells do not form tumours in nude mice in our experiments and have a colony-forming efficiency in agar of 3%. The problem of identification encountered here illustrates the usefulness of directly testing material such as red cells, white cells, or in these cases samples of the tumours from the original donors.

The reliability of enzymes as genetic markers for cell identification depends on the stability of the enzyme phenotypes in culture. We have attempted to check this particular point by repeatedly testing cells in culture over various periods of time. Whenever possible, material from the original donor has also been examined. We have monitored more than 100 lymphoid lines from more than

Table 2 Enzyme phenotypes in five bladder tumour lines and some different strains of HeLa

Enzyme	Bladder tumours					HeLa	HeLa _{s3}	HEP-2	D98	FLA	BU25	KB	LU	EUE
	T24	RT4	J82	RT112	EJ									
PGM ₁	1	2-1	1	1	1	1	1	1	1	1	1	1	1	1
PGM ₂	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PGM ₃	1	2-1	1	2-1	1	1	1	1	1	1	1	1	1	1
GOT _M	1	1	1	2-1	1	1	1	1	1	1	1	1	1	1
ESD	1	2-1	1	2-1	1	1	NT	1	1	NT	NT	NT	NT	NT
AK ₁	1	1	1	1	1	NT	NT	NT	1	NT	1	1	1	1
ADA	1	1	1	1	1	1	1	NT	1	NT	1	1	1	1
ACP ₁	BA	A	B	B	B	NT	BA	NT	BA	NT	BA	BA	NT	NT
PGD	A	A	A	A	A	A	A	NT	A	A	A	A	A	A
G6PD	B	B	B	B	B	A	A	A	A	A	A	A	A	A
PEPA	1	1	1	1	1	1	1	1	1	NT	1	1	1	1
PEPC	1	1	1	1	1	1	1	1	1	NT	1	1	1	1
ACON _s	NT	NT	NT	NT	NT	1	NT	NT	1	NT	NT	NT	NT	NT

In addition to the enzymes shown here D98 was tested for lactate dehydrogenase (LDH_A and LDH_B), mannose phosphate isomerase (MPI), superoxide dismutase (SOD_A), soluble and mitochondrial malate dehydrogenase (MDH_S and MDH_M), isocitrate dehydrogenase (ICD_S), purine nucleoside phosphorylase (NP), glucose-phosphate isomerase (GPI), adenine phosphoribosyl transferase (APRT), enolase (ENO₁), glyoxalase I (GLO) and mitochondrial malic enzyme (ME_M). It seemed to have the common homozygous phenotype at all these loci with the exception of ME_M, for which no satisfactory result was obtained. However, two other HeLa strains tested for ME_M (HELA_{s3} and LU) were clearly heterozygotes, phenotype ME_M 2-1.

70 people, about 100 primary fibroblast cultures and a small number of permanent epithelial lines in addition to those described here. From one lymphoid line more than 500 clones were examined, each for an average of 30 loci, 300 of these clones having been treated with large doses of mutagen (ref. 24 and further unpublished data). The total number of tests performed has been more than 20,000, since many lines have been checked repeatedly. In all these cells only two examples of a true mutation, involving alteration of electrophoretic mobility, have been seen (ref. 23 and unpublished work). Loss of enzyme activity, often involving only one allele so that an apparent homozygote is produced, has also been observed but with very low frequency²⁴. In several of these cases this was associated with a specific chromosomal aberration²⁵. On 15 other occasions an apparent change in phenotype was observed, three times in fibroblast cultures and 12 times in lymphoid lines. These changes were conclusively shown to be due to contamination by checking other enzymes and by karyotype analysis.

Clearly more extensive investigations into the identity of cell lines would be desirable, and other workers have suggested HLA typing²⁶ and monitoring of the karyotype, particularly for the presence or absence of a Y chromosome⁶. Both of these methods have many advantages which we do not wish to ignore. We feel, however, that the routine testing of enzyme phenotypes, while perhaps not yet equalling the theoretical discriminating power of the HLA system, is such a reliable and simple tool for the characterisation of human cultured cells that it should be adopted with much greater enthusiasm than hitherto.

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Phenotypic reversion of ricin-resistant hamster fibroblasts to a sensitive state after coating with glycolipid receptors

THE cytotoxicity of the plant lectin ricin, of *Ricinus communis* seeds, towards mammalian cells is mediated in three steps¹. Binding of the toxin to cell surface galactose or N-acetylgalactosamine receptors is followed by entry of the bound lectin into the cells, perhaps by endocytosis^{2,3} and inhibition of protein synthesis⁴⁻⁶. Several variant lines of cells selected for resistance to ricin have been shown⁷⁻¹⁰ to bind ricin poorly and to carry much smaller amounts of receptors at the cell surface. We report here that when such a resistant cell line of baby hamster kidney (BHK) fibroblasts^{8,9} is incubated with a ricin-binding glycolipid fraction prepared from human erythrocytes, glycolipid is taken up and the cells exhibit increased binding of ricin and greater sensitivity to lectin-mediated inhibition of cell protein synthesis. These studies suggest that glycolipids can function in certain circumstances as membrane receptors in the binding of ricin and mediation of ricin cytotoxicity.

Baby hamster kidney (BHK) cells and the ricin-resistant variant, Ric^h21 were grown at 39 °C in Glasgow modified Eagle's medium (GMEM) supplemented with 10% tryptose phosphate and 10% foetal bovine serum as described previously^{8,9}.

A ricin-binding glycolipid fraction was isolated from human blood group O erythrocytes as described before¹¹⁻¹³. The fraction soluble in chloroform:methanol:water (1:2:1) containing fucose (8%), galactose (41%), glucosamine (33%), fatty acids (4%) and sphingosine (5%) was evaporated to dryness just before use, dissolved in water at 5 mg per ml and the resulting transparent aqueous solution was sterilised by filtration. ¹⁴C-labelled glycolipid was prepared by de-N-acetylation in N-sodium hydroxide at 100 °C for 30 min and reacylation with ¹⁴C-acetic anhydride after neutralisation and dialysis. The specific activity was 1.3 × 10⁶ c.p.m. mg⁻¹.

As Fig. 1 shows, when cells were incubated in phosphate-buffered saline (PBS) with ¹⁴C-labelled glycolipid about 2% of the labelled glycolipid was taken up in cell-bound form regardless of the initial concentration of glycolipid added. Maximum uptake by cells required 4 h of incubation with the glycolipid fraction at 39 °C. Measurable binding did not occur at 2 °C. Cells incubated for 4 h at 39 °C with glycolipid (50 µg ml⁻¹) incorporated, in several independent experiments, 5–12 × 10⁶ molecules per cell. At this time no radioactivity was released from the cells by brief trypsinisation (2 µg ml⁻¹ at 39 °C for 5–15 min) indicating that the cell-bound glycolipids were not loosely attached, for example, to serum proteins adsorbed at the cell surface.

Incorporated glycolipid increased the binding of radioiodinated ricin to normal BHK cells as well as to the ricin-resistant variant, Ric^h21 (Fig. 2). With cells containing 5 × 10⁶ molecules of glycolipid per cell, ricin binding was enhanced to a similar extent by approximately 3 × 10⁶ sites per cell. The enhancement was most marked with Ric^h21 cells which by themselves show only about 10% of the ricin-binding capacity of normal BHK cells (Fig. 2). After glycolipid treatment (50 µg ml⁻¹) ricin binding was increased threefold. The enhancement of ricin binding was

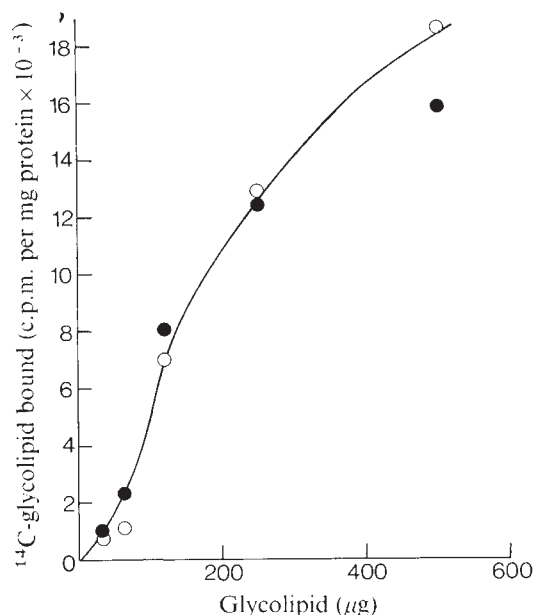


Fig. 1 Binding of ^{14}C -labelled human erythrocyte glycolipids to BHK cells. Confluent monolayers of parental BHK cells (●) or ricin-resistant variant (○), Ric^R21, on 35-mm diameter plastic dishes (4×10^6 cells per dish) were exposed in 1 ml of PBS to various concentrations of the ^{14}C -labelled glycolipid fraction at 39 °C for 4 h. The saline was removed, the cells were washed three times with saline and then solubilised in 1 ml of N-NaOH and counted for bound radioactivity. Other portions were assayed for protein.

greater, about sixfold, after treatment of Ric^R21 cells with glycolipid at $500 \mu\text{g ml}^{-1}$, and ricin binding then approximated to that of normal BHK cells. Ricin binding to normal BHK cells was doubled after similar treatment. But the enhancement of ricin binding, 6.7×10^6 sites per cell, was considerably less than the numbers of glycolipid molecules bound to the cells after treatment with $500 \mu\text{g}$ of glycolipid. We conclude that cells exposed to very high concentrations of glycolipids ($500 \mu\text{g ml}^{-1}$) take up glycolipids either intracellularly or in surface sites that are not accessible to ricin added externally. Perhaps in these conditions glycolipids in part were sequestered intracellularly by pinocytosis. We do know, however, that the glycolipids used in this work can be incorporated into erythrocytes in which pinocytosis is improbable, and confer Lewis and ABO-blood group activity on these cells¹³.

Whatever the mechanism of incorporation¹⁵, treatment of BHK cells with glycolipids, in conditions in which an association with the cell surface could be demonstrated by an enhancement of ricin binding, was found to increase the sensitivity of the cells to ricin. Ricin-mediated inhibition of cell protein synthesis was measured as follows. Confluent monolayers of normal BHK cells or ricin resistant Ric^R21 variant cells growing on 35-mm diameter plastic dishes were incubated in GMEM minus serum (1 ml) with ricin ($1 \mu\text{g}$ or $10 \mu\text{g}$). After 1 h at 39 °C, 1 ml of ^3H -leucine ($3.3 \mu\text{Ci}$, 59 Ci mmol^{-1}) in leucine-free GMEM was added to each dish. Incorporation was carried out over 1–6 h when the medium was removed and the cell monolayers were washed three times with PBS, twice with cold (2 °C) 2% phosphotungstic acid 10% perchloric acid and twice with cold alcohol. The washed monolayers were then dissolved in hot (80 °C) N-NaOH

Table 1 Inhibition of BHK cell protein synthesis by ricin

Experiment	Cells	Glycolipids ($\mu\text{g ml}^{-1}$)	Ricin ($\mu\text{g ml}^{-1}$)	^3H -leucine incorporation (c.p.m. per mg protein)	Incorporation (% control)
1	Normal BHK	None	0	64,000	100
			1	13,400	21
			10	7,050	11
		50	0	69,000	100
			1	6,950	10
			10	7,510	12
		500	0	53,360	100
			1	5,810	11
			10	2,660	5
	Ric ^R 21	None	0	77,500	100
			1	78,350	196
			10	75,800	98
		50	0	78,300	100
			1	76,000	97
			10	37,600	48
		500	0	73,500	100
			1	49,300	67
			10	17,400	23
2	Ric ^R 21	None	10	212,000	100
		125	10	75,500	37
		250	10	74,000	33
		500	10	47,000	22
		500	10*	172,000	86
3	Ric ^R 21	None	10	170,000	100
		50	10	127,000	74
		200	10	68,000	40
		50†	10	153,000	90
		200†	10	156,000	92

Cells were incubated for 4 h at 39 °C with or without erythrocyte glycolipid fraction as indicated. The cells were then washed and incubated with ricin ($1 \mu\text{g ml}^{-1}$ or $10 \mu\text{g ml}^{-1}$) for 1 h at 39 °C before assay of ^3H -leucine incorporation into cellular protein. Control dishes contained no ricin and showed that treatment of cells with glycolipid had no effect on the level of ^3H -leucine incorporation into protein.

*This solution was supplemented with 10 mM lactose.

†A degraded glycolipid derivative which shows no ricin-binding activity was prepared¹³ by Smith oxidation with 40 mM sodium periodate in 50 mM sodium acetate buffer, pH 4.5 overnight at 4 °C to destroy galactose terminal units. Excess oxidant was destroyed with ethylene glycol and dialdehydes were reduced with sodium borohydride at room temperature for 1 h. Excess borohydride was destroyed by acetic acid and acetal linkages were hydrolysed in 0.5 N HCl at 20 °C for 8 h. Finally the degraded glycolipid fraction was dialysed extensively against water and freeze dried. The material was then dissolved (5 mg ml^{-1}) in water.

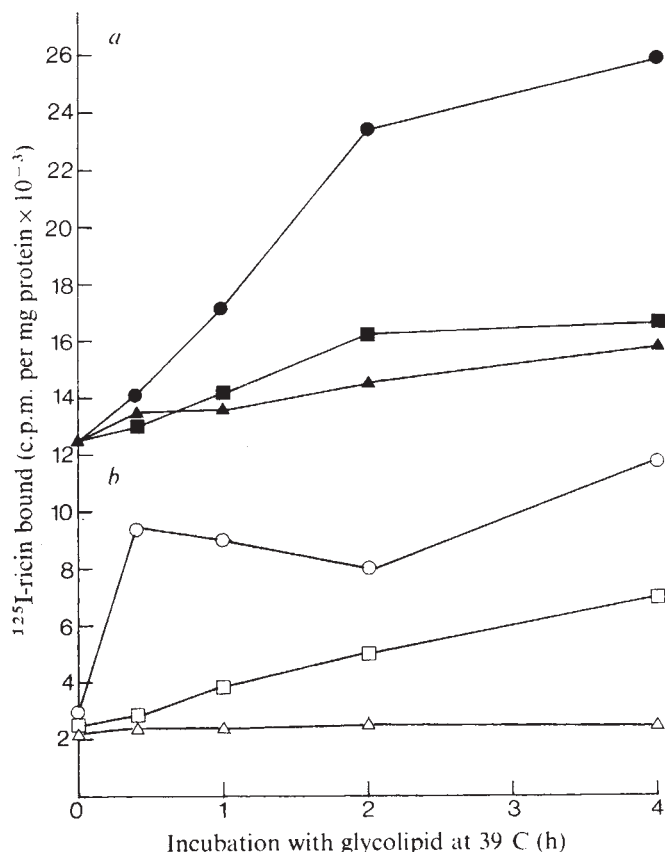


Fig. 2 Binding of ^{125}I -ricin to BHK cells before and after treatment with erythrocyte glycolipids. Normal cells (a) and ricin-resistant variant $\text{Ric}^{\text{R}21}$ (b) were grown to confluency on 35-mm diameter plastic dishes (4×10^6 cells per dish). Growth medium was removed and duplicate monolayer cultures were incubated at 39°C with $50\text{ }\mu\text{g}$ or $500\text{ }\mu\text{g}$ of glycolipid in 1 ml of PBS. Control dishes were incubated with PBS. At various times, unbound glycolipid was removed by washing three times with saline and ^{125}I -ricin ($50\text{ }\mu\text{g}$, $3.5 \times 10^5\text{ c.p.m.}$) in PBS (1 ml) added at 2°C . After incubation of control cells and glycolipid-treated cells with ricin for 60 min at 2°C the monolayers were washed three times with saline, dissolved in 1 ml N-NaOH and counted. Ricin binding is expressed in terms of cellular protein and corrected for non-specific binding of ^{125}I -ricin to cells (control or glycolipid-treated) in the presence of hapten inhibitor (10 mM lactose) and to dishes containing no cells to which appropriate amounts of glycolipid had been added, incubated at 39°C for the stated times and then washed with saline. In no case did the nonspecific binding (lactose irreversible counts or counts bound to dishes) exceed 20% of the radioactivity bound in the absence of lactose to cell layers in identical conditions. $\bullet$ and $\circ$, $500\text{ }\mu\text{g}$ glycolipids; $\blacksquare$ and $\square$, $50\text{ }\mu\text{g}$ glycolipids; $\blacktriangle$ and $\triangle$, controls.

(1 ml) and portions (0.2 ml) were removed for radioactive counting and protein assays. Incorporation of ^3H -leucine into protein by normal BHK cells was inhibited about 80% by ricin at $1\text{ }\mu\text{g ml}^{-1}$ and 90% by ricin at $10\text{ }\mu\text{g ml}^{-1}$ (Table 1, experiment 1). In contrast, protein synthesis of the resistant variant, $\text{Ric}^{\text{R}21}$, was unaffected by these concentrations of ricin (Table 1, experiment 1). As Table 1 shows, in $\text{Ric}^{\text{R}21}$ cells incubated with glycolipids for 4 h at 39°C and then, after washing, with ricin ($1\text{ }\mu\text{g}$ or $10\text{ }\mu\text{g}$) for 1 h , the incorporation of ^3H -leucine into protein was decreased significantly compared with cells not pretreated with glycolipids. The already high sensitivity of normal BHK cells to these concentrations of ricin also appeared to be significantly increased by pretreating cells with glycolipids (Table 1, experiment 1). The magnitude of the effect of $\text{Ric}^{\text{R}21}$ cells was dependent on glycolipid concentration to some extent (Table 1, experiment 2). Furthermore, the effect of ricin on $\text{Ric}^{\text{R}21}$ cells pretreated with glycolipids was abolished by inclusion

of 10 mM lactose in the culture medium (Table 1, experiment 2). Additional evidence that the potentiation of ricin cytotoxicity by pretreatment of $\text{Ric}^{\text{R}21}$ cells with glycolipids requires binding of lectin to the carbohydrate moiety of the glycolipids was obtained using a degraded glycolipid fraction (Table 1, experiment 3). Oxidation of the glycolipid fraction with periodate destroys preferentially the ricin-binding terminal galactose residues of the molecule¹³. When this material was incorporated into $\text{Ric}^{\text{R}21}$ cells, there was no change in the high resistance of the cells to ricin (Table 1, experiment 3).

We conclude from our results that a ricin-binding glycolipid fraction extracted from human erythrocytes when added to baby hamster kidney fibroblasts becomes tightly associated with the cells. Such incorporation increases the numbers of toxin molecules that can bind to the cells and the cells concurrently become considerably more sensitive to the cytotoxic action of ricin as indicated by a decreased incorporation of ^3H -leucine into cellular proteins. It is unlikely that complex glycolipids containing twenty or more monosaccharides and conferring blood group specificity on human erythrocytes exist endogenously in hamster fibroblasts. But other ricin-binding glycolipids, for example ganglioside GM1 (ref. 14), are known that could function as the normal receptors mediating ricin toxicity in sensitive BHK cells. Preliminary evidence shows no detectable levels of GM1 or GM2 in either normal BHK cells or $\text{Ric}^{\text{R}21}$ cells, however, and the levels of other galactolipids, GM3 (hematoside) and lactosylceramide, are similar in the two cell lines. We therefore favour the alternative interpretation that incorporation of relatively large amounts of "foreign" glycolipids into the BHK surface membrane can mimic the normal route, for example through glycoprotein receptors, by which the toxin enters these cells.

It is clear that the incorporated glycolipids are less efficient in mediating ricin toxicity than are the natural receptors. Thus, $\text{Ric}^{\text{R}21}$ cells that bound sufficient glycolipid to enhance ricin binding to approximately the level exhibited by normal BHK cells (6×10^6 sites per cell) were only about half as sensitive to ricin (Table 1, experiment 1). The mechanism by which glycolipid incorporated into the surface membrane participates in ricin cytotoxicity is, of course, unknown. It may do so directly, or by association with integral glycoproteins, perhaps those that in normal cells carry the ricin-binding sites. We have no unequivocal way of deciding between these two mechanisms, neither of which would be expected to be as efficient as the normal process. In the latter case, it may be that the association of glycolipid with putative glycoprotein mediators of ricin toxicity involves only a small proportion of the total glycolipid incorporated into the cell membrane. Perhaps only when the membrane is heavily loaded with glycolipid does the number of glycoprotein molecules "primed" with glycolipid receptors reach a threshold value necessary for permeation of ricin into the cells.

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Cellular adhesiveness reduced in ricin-resistant hamster fibroblasts

THE means by which tissue cells adhere to one another and to non-cellular surfaces is not known, although complex carbohydrate of their surface membrane may be involved^{1,2}. To test this, one can ask whether mutations which affect

the structure of surface carbohydrate also alter cellular adhesiveness. Meager *et al.*³ have isolated a series of stable variants of baby hamster kidney fibroblasts (BHK 21, clone 13) which are resistant to the toxic lectin ricin. The first step in killing of cells by ricin is binding of the lectin to galactosyl- or *N*-acetylgalactosaminyl residues at the cell surface^{4,5}, and many ricin-resistant clones yield cell lines with reduced glycosylation of their cell surfaces³. We now report that, judged by their ability to form aggregates in suspension, the intercellular adhesiveness of a number of ricin-resistant lines is less than unselected lines. We have also found that cells of most lines attach less readily than controls to glass coverslips bearing adsorbed films of serum or gelatin.

After 24 h of incubation in a gyratory shaker⁶, suspensions of control cells yield compact spherical aggregates approximately 200 μ m in diameter (Fig. 1). Ricin-resistant

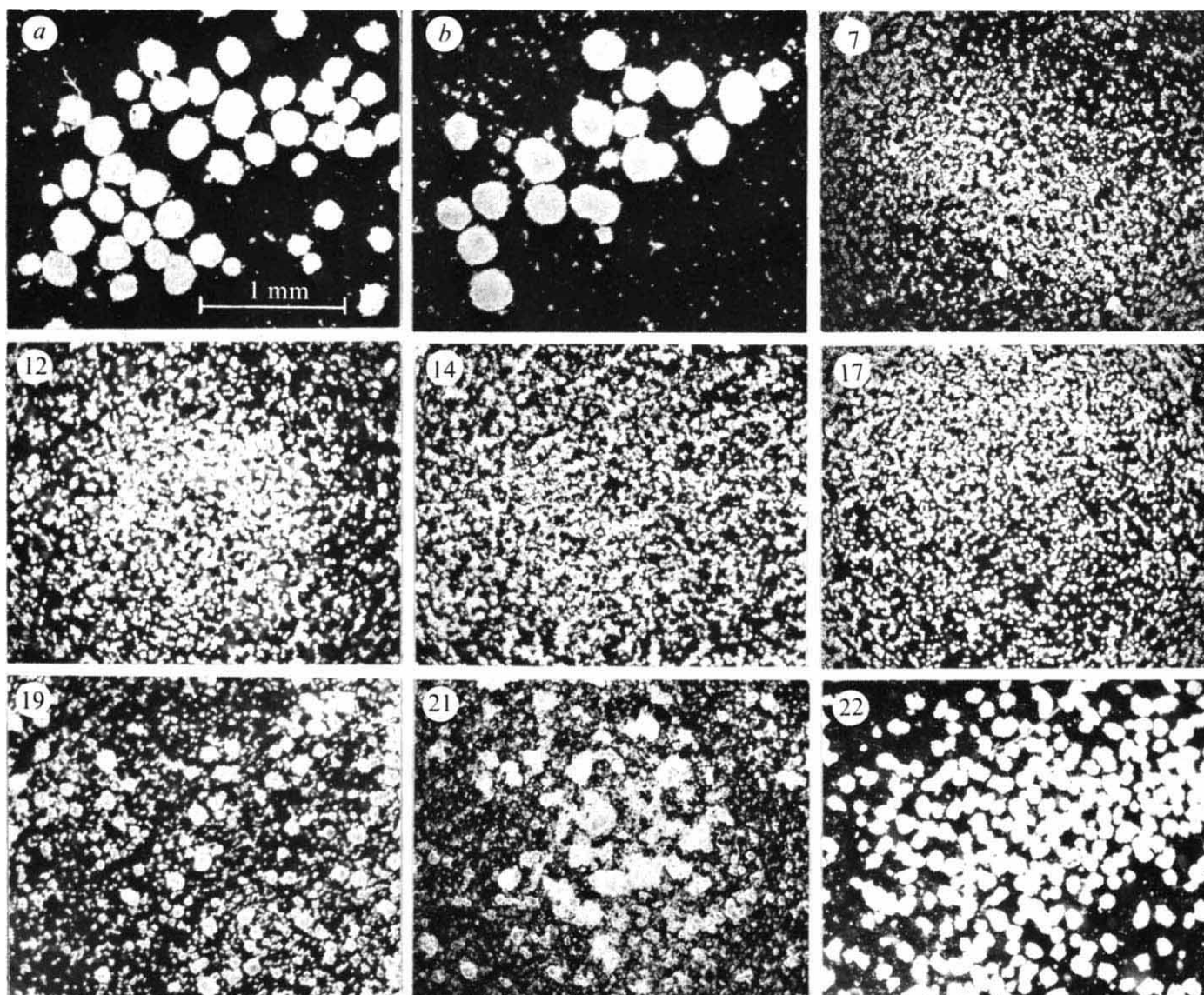


Fig. 1 Darkground micrographs of aggregated suspensions of BHK 21 cells and ricin-resistant derivatives after gyratory shaking for 24 h. *a*, C13 cells from which ricin-resistant cells were selected. *b*, Mutagenised but unselected control (see below) and resistant lines as numbered. Confluent cultures grown in Glasgow-modified Eagle's medium supplemented with 10% foetal bovine serum and 10% tryptose phosphate (EFT) were dispersed using EDTA and trypsin¹⁷. Cells were resuspended in EFT at 5×10^5 per ml. 2 ml of each suspension was cultured in a 25-ml silicone-treated conical flask, with gas phase air, 95%, CO₂ 5%, in a gyratory shaker⁶ at 70 r.p.m. 37 °C for 24 h. 0.25-ml samples were photographed in a dish, diameter 14 mm. C13 cells from which ricin-resistant cells were selected at Mill Hill ((*a*) in Figs 1-3) showed adhesive properties very similar to Glasgow stocks used in earlier experiments^{8,17}. Two further control lines were obtained by mutagenising C13 cells as for selection, but then growing out the subclones in the absence of ricin. Our unpublished experiments show that cells of lines Ric¹⁴, 19 and 21, but not 22, have lost the ability to readhere in the early phase of aggregation which occurs in the first hour of shaking of freshly suspended cells¹⁷. This assay is not satisfactory for comparing lines selected from C13 cells, because it is relatively easy to obtain from C13 stocks without selection, lines which do not aggregate in the first hour from trypsin dispersal¹⁸. Lines of this phenotype do however aggregate very similarly to unrecycled C13 cells when cultured for 24 h in a gyratory shaker.

(Ric^R) lines exhibit a range of reduced aggregation behaviour, from Ric^R22 which differs from controls only in forming smaller aggregates, to Ric^R7 and 17 which aggregate very little. Quantitation by use of a Coulter counter shows that these comparisons are repeatable and stable through a month's consecutive passage of the cells (Fig. 2) and after storage of cells in liquid nitrogen for 1 yr. Figure 3a shows that freshly dispersed cells of Ric^R19 and 22 adhere to a film of adsorbed foetal bovine serum to the same extent as controls, whereas the remaining six tested adhere less well. This behaviour is not specific to foetal calf serum, for the pattern is similar with calf serum and gelatin (Fig. 3b), although there is much more day to day variation on these latter films.

Ric^R22 cells exhibit at confluence the parallel alignment characteristic of the parental cells, bind normal levels of ricin^{3,7} and as shown here, also resemble wild type in adhesive behaviour. This agrees well with the evidence that the moderate resistance to ricin of this line depends on a cytoplasmic, rather than a surface alteration⁷. Ric^R12, 16 and 19 are lines which do not show a reduction in ricin binding, but nevertheless may have reduced surface glycosylation, since they are cross resistant to the lectin from *Phaseolus vulgaris*³. The remaining lines, including those

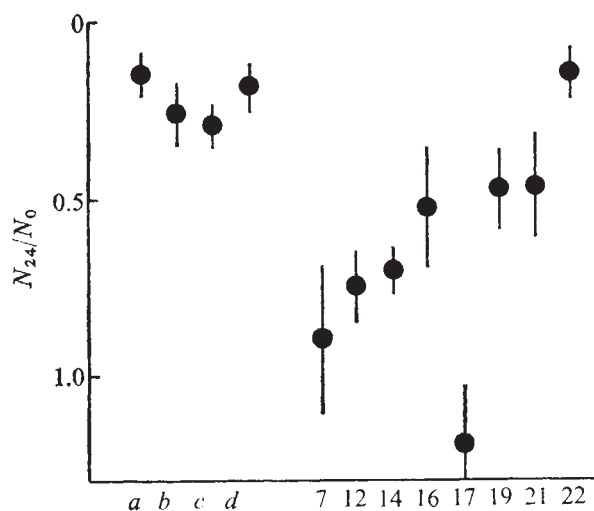


Fig. 2 Aggregation of suspensions of BHK 21 cells and ricin-resistant derivatives measured using an electronic particle counter. *a*, C13 cells from which ricin-resistant cells were selected; *b* and *c*, mutagenised but unselected lines; *d*, Glasgow stocks of C13 cells. 0.1-ml samples of suspensions aggregated as for Fig. 1 were diluted in 20 ml of 0.9% w/v sodium chloride and counted in a Coulter counter Z_B, using a 200- μ m aperture⁴. Data are the concentration at 24 h of particles of all sizes divided by the value before incubation. For each cell line, means and standard deviations are shown from six to eight separate experiments, spread through about eight consecutive passages of the cells. The count does not reflect the tendency of Ric^R22 to form smaller aggregates than wild-type cells since the assay is dominated by the proportion of particles representing single cells and very small clusters. Values greater than 1 for Ric^R17 indicate limited cell division in the cultures.

most conspicuously affected in aggregation (7 and 17) all bind only 10–40% as much ricin as do wild type³. The aggregation properties of these lines are therefore broadly consistent with the proposal that intercellular adhesion requires carbohydrate chains which are potential acceptors of terminal sialyl residues^{8–10}. Indeed this 'sialyl acceptor' model predicts a strong correlation between ricin resistance and decreased intercellular adhesiveness, for those monosaccharide residues often terminal in sialic-acid free carbohydrate chains (galactose or *N*-acetylgalactosamine), are the same as those required for ricin binding. Besides these residues, the model requires the existence of a cell-surface protein with carbohydrate-binding activity (perhaps a sialyl transferase² or lectin-like protein¹¹) of specificity resembling

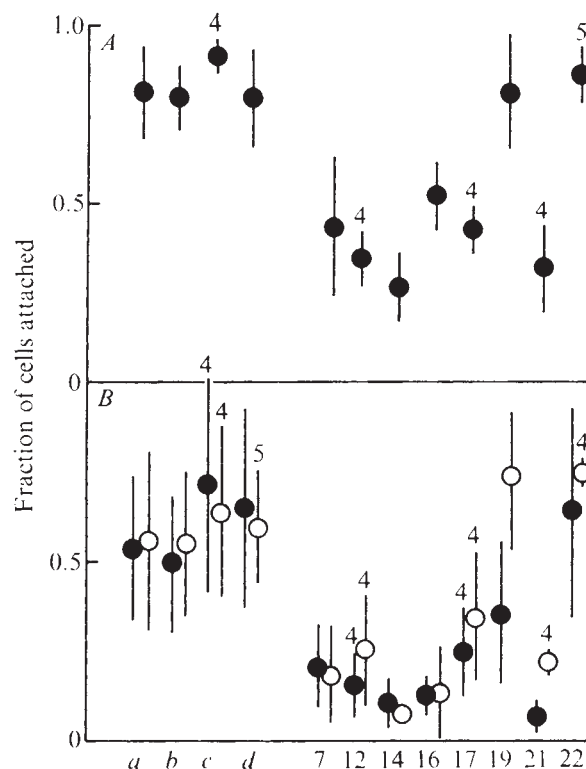


Fig. 3 Attachment of BHK 21 cells and ricin-resistant derivatives to glass coverslips bearing adsorbed protein films. *A*, foetal bovine serum; *B*, calf serum (solid symbols) gelatin (open symbols). *a–d* as Fig. 2. Data are the fraction of cells which had attached after 45 min. Mean and standard deviation of 4–14 experiments on each line, number shown where less than six. Attachment was measured much as described by Rabinovitch and De Stefano¹⁹ except that cells were labelled by incorporation of ³²P-phosphate. Cells were grown for 24 h in medium minus tryptophan phosphate (EF) containing 2 μ Ci of ³²P-phosphate per ml, which was then replaced with unlabelled EFT for a further 18 h growth. Cells dispersed with EDTA and trypsin were resuspended in Hanks medium, pH 7.2, buffered with 0.01 M HEPES medium at 10⁶ cells per ml. Protein-coated coverslips¹⁸, diameter 13 mm, were drained but not allowed to dry, immediately before receiving 0.1 ml of cell suspension. After incubation for 45 min in a humid atmosphere at 37 °C, the coverslips were rinsed in Hanks-HEPES, drained and transferred directly to scintillation phials for counting without further processing. Data are c.p.m. retained as a fraction of total c.p.m. in 0.1 ml cell of suspension, from triplicate coverslips in each experiment.

ricin. We have found that homogenates of BHK cells contain a haemagglutinin selectively inhibited by *N*-acetylgalactosamine and D-fucose¹², but we do not yet know if it is involved in the adhesive phenomena described here. The sialyl-acceptor model does not readily explain the simultaneous effect of the mutations in most lines on both intercellular adhesion and adhesion to substratum. Possibly carbohydrate binding is involved in some purely *cis* interaction (such as clustering in the plane of the surface of a glycoprotein involved in adhesion) rather than, or in addition to, a *trans* role (Fig. 4). Alternatively, one of the interacting components could simply adsorb to the substratum¹³. We should stress, however, that there is no direct evidence that protein carbohydrate 'recognition' is involved in any adhesive properties of these cells. Indeed interpretation of the adhesive changes as a direct result of decreased cell-surface glycosylation may yet prove to be an oversimplification to, a *trans* role (Fig. 4). Alternatively, one of the inter-resistant BHK cell lines lacks a glycoprotein of approximate molecular weight 250,000 which can be labelled by lactoperoxidase-catalysed iodination¹⁴, and such surface components may themselves be involved in cell adhesion^{13,15}.

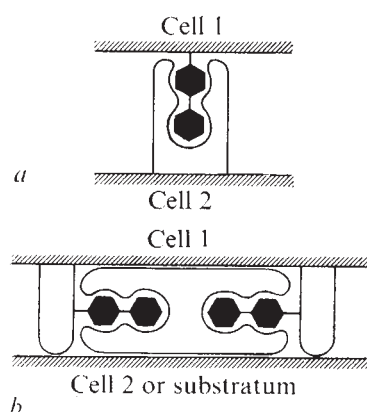


Fig. 4 Possible roles for carbohydrate-protein binding in cell adhesion. *a*, Sialyl-acceptor model⁸, in which an oligosaccharide (solid hexagons) of cell-surface carbohydrate, with terminal galactose or *N*-acetylgalactosamine is bound by a specific receptor on a second cell. *b*, Alternative interaction in which a similar receptor system is involved in aggregation of a glycoprotein at regions of cell-cell or cell-substratum contact.

In lines such as Ric¹⁴ which show lowered adhesiveness, and yet seem to contain normal levels of this component, the glycoprotein could bear a structural alteration affecting its function in adhesion, but not its presence at the cell surface.

Finally, although many ricin-resistant BHK cell lines resemble in adhesive properties certain lines of virus transformed cells¹⁶, none of the lines used in these experiments exhibits a transformed phenotype with respect to growth, since they grow poorly in low serum and in agar suspension, and yield saturation densities similar to the parental line.

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Haemopoietic stem cells are organised for use on the basis of their generation-age

THE spleen-colony assay of Till and McCulloch¹, which is a clonal assay², measures the content of haemopoietic cells in blood-forming tissues. Stem cells (colony forming units in spleen, CFUS) form red cells, granulocytes, platelets and themselves (self-renewal) in these colonies^{3,4}. Worton *et al.*⁵ reported that stem cells separated by velocity sedimentation differed in their capacity for self-renewal. Schofield and Lajtha⁶ reported that CFUS from mice treated with isopropyl methane sulphonate have a low self-renewal capacity. We have studied the functional capacity of myeloid stem cells from normal mice (NBM) and mice treated with hydroxyurea. CFUS from mice treated with five injections of hydroxyurea (5HUBM, G.S.H. and N. M. Blackett, unpublished) formed 3 times more CFUS and 2.5 times more colony forming units in culture per original colony (CFUC, a committed precursor of granulopoiesis⁷) than those from NBM. When these grafted CFUS were re-transplanted they still formed three times more CFUC and CFUS per secondary colony than those from NBM (Tables 1 and 2).

Table 1 Cellular products of the first graft of NBM and 5HUBM

Group	Colonies per spleen ± s.e.m.	CFUC per spleen ± s.e.m. ($\times 10^{-3}$)	CFUC per colony ± s.e.m. ($\times 10^{-3}$)	CFUS per original colony
IC	0.3 ± 0.3	0		
NBM	34.8 ± 1.3	1.46 ± 0.34	4.23 ± 1.0	43.2 ± 2.3
5HUBM	51.3 ± 1.9	5.53 ± 0.61	10.84 ± 1.26	128.2 ± 8.3

NBM donors were 10 normal 3-month BALB/c female mice. 20 similar mice received hydroxyurea (Calbiochem) intraperitoneally (1 mg per g body weight) -32, -26, -10, -7 and -2 h before collection⁸. These were 5HUBM donors. Recipients were 2.5-3-month-old male and female BALB/c (20 mice per group). They were irradiated before grafting (700 R, 230 kVp (kilovolt peak)), HVL (half value layer) 0.5 mm Cu, 146 R.min., TSD (target-source distance) 50 cm., Maximar). Ten days after grafting half the recipients in each group were killed. Their spleens were fixed in formalin-acetic acid-ethanol (1:0.5:8.5) and the surface colonies counted. Twelve days after grafting the remaining recipients were killed. Their spleens were collected aseptically, passed through sterile stainless steel sieves and gauze and assayed individually for CFUC and in pooled groups for CFUS (20 recipients per group). CFUC were cultured in sealed, humidified boxes and counted on day 14. Control dishes with NBM (and the same source of colony-stimulating factor) formed 400 colonies per 10^4 nucleated cells.

An hypothesis to explain these differences proposes that the use of haemopoietic stem cells to satisfy haemopoietic stress is based on the generation-age of these cells. A young stem cell is one that has undergone few generations since its origin, consequently its capacity for self-renewal and the production of committed precursors is greater than that of an older stem cell which has undergone more generations. Consider the differences between 5HUBM and NBM in the light of this proposal. The effect of the first two doses of hydroxyurea is to strip the haemopoietic tissues of dividing progenitors⁸ and, consequently, of their products. As a result CFUS move into cycle and are, in their turn, killed by further injections of hydroxyurea. It is assumed that the normal regulatory mechanisms bring the more mature CFUS from the pool of CFUS into cycle. On d 2 of treatment with hydroxyurea those cells in the S phase of the cell cycle are killed and the rest gathered at the G₁-S border by injections at -10 and -7 h. As the drug is cleared the blocked cells move into S and are killed by the fifth dose of hydroxyurea at -2 h. Those cells which survive the fifth dose of hydroxyurea are functionally more efficient than those which have survived four injections. (Splenic DNA and blood ⁵⁹Fe uptake are more than 1.5 times greater and

Table 2 Cellular products of retransplanted stem cells from NBM and 5HUBM

Group	Cell dose in splenic equivalents of the primary graft	Colonies per spleen $\pm$ s.e.m.	CFUC per spleen $\pm$ s.e.m. ($\times 10^{-5}$)	CFUC per secondary colony $\pm$ s.e.m. ($\times 10^{-3}$)	CFUS per secondary colony $\pm$ s.e.m.
IC	0.03	3.9 ± 1.3	0.01 ± 0		
NBM	0.03	53.6 ± 1.2	0.78 ± 0.13	1.6 ± 0.3	17.1 ± 1.4
5HUBM	0.006	47.5 ± 1.9	2.04 ± 0.24	4.7 ± 0.6	51.8 ± 3.9

Experimental conditions were the same as those in Table 1 except that NBM controls in the CFUC assay formed 150 colonies per 10^4 nucleated cells.

CFUC per colony almost 2 times greater (M.R., unpublished results.)

The hypothesis predicts that CFUS from foetal liver will have greater capacity for self-renewal than those from NBM and that stem cells, such as those in the blood whose capacity for self-renewal is less than that of NBM⁹, can be shown to be "older". Preliminary results confirm that this is so.

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Cellular and humoral immune responses in vaccination against dental caries in monkeys

PROTECTION against dental caries in the rhesus monkey by immunisation has now been characterised with respect to the development of *Streptococcus mutans*¹ and the corresponding antibodies²⁻⁴. One of the essential requirements for protection is the development of an optimum level of antibodies, at a critical time, before a large enough number of *Strep. mutans* has developed to initiate caries formation². Antibody synthesis, amplification mechanisms and protection depend on cellular interactions associated with T and B lymphocytes and macrophages⁵. The aims of this investigation were to study the part that cell-mediated immune responses might play in the protective mechanism against dental caries. The incidence of caries and the growth of *Strep. mutans* in rhesus monkeys was studied sequentially and compared with the development of serum antibodies, *in vitro* DNA synthesis of antigen stimulated lymphocytes, leukocyte migration inhibition (LMI) and *in vivo* skin delayed hypersensitivity (DH) to *Strep. mutans*.

Eight young rhesus monkeys were caged, examined and maintained on a human type of diet, containing 15% sucrose⁶. All animals had a fully erupted deciduous dentition and the permanent first molars were erupted in four monkeys; in the other four the permanent first molars erupted into a functional position within 30 weeks of the start of the experiment. As younger animals tend to develop more caries^{7,8}, the distribution of the monkeys was biased against the immunised group by including three of the younger monkeys without the permanent molars. A strain of

Strep. mutans (serotype c) was isolated from the dental plaque of a rhesus monkey which was used as a control in a previous experiment¹. The organism was grown in Todd-Hewitt broth, killed with 0.6% formalin, and a vaccine containing 2×10^9 *Strep. mutans* per ml was prepared². The three youngest monkeys were given subcutaneous injections of the vaccine mixed with an equal volume of Freund's incomplete adjuvant; 0.5 ml was injected into the right arm and 0.5 ml into the left leg at the start of the experiment. The same amount of *Strep. mutans* in saline was administered subcutaneously 30 weeks later. The sham-immunised group of three monkeys was given subcutaneous injections of 0.5 ml of saline instead of the vaccine. A further control group of two monkeys had no injections.

The animals were examined clinically and by means of X-rays for the presence of dental caries, they were weighed and blood was taken from the femoral vessels at 3-weekly intervals. At the same time, dental plaque was collected with a probe from the cervical and approximal surfaces of the upper left deciduous molars. The two control groups showed a very similar increase in caries and the number of colony-forming units (CFU) of *Strep. mutans*, so that they were combined for statistical analysis. Smooth surface (SS) caries was detected from week 18 (Fig. 1) and the mean rate of increase in caries in the immunised animals was significantly less than in the controls ($P < 0.05$). At week 54 a mean of $8.4 (\pm 1.2)$ cavities per animal was found in the controls compared with 2.7

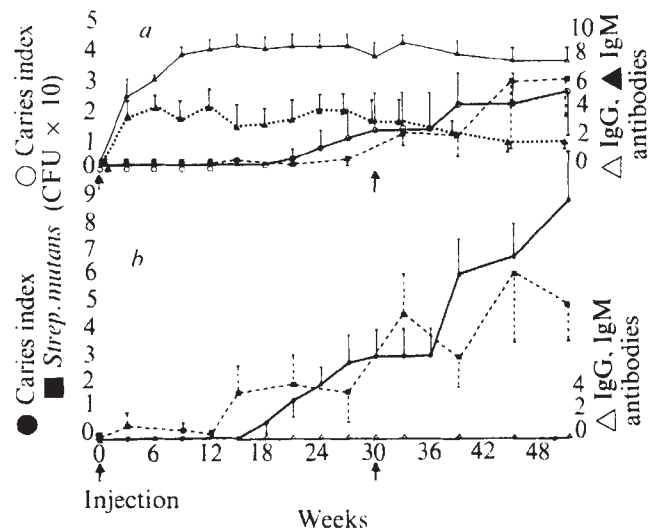


Fig. 1 Sequential analysis of caries, antibodies and *Strep. mutans* in monkeys immunised with *Strep. mutans*. For antibody titration¹⁸ doubling dilutions of sera were added to air-dried smears of cells of the immunising *Strep. mutans* for 30 min, washed three times with phosphate-buffered saline (PBS), and then overlaid with anti-human IgG (F:P 3:1), or IgM (F:P 4:1) fluorescein-isothiocyanate conjugates (Wellcome). The reagents were checked for class specificity and cross reactivity with monkey IgG and IgM and specificity of the test was established by complete absorption of the antibody titre with the homologous and little or no absorption with heterologous organisms. The titres were expressed as $\log_2(1:1.5)$. *Strep. mutans* CFU are expressed as the percentage of total anaerobic colonies on TYC agar¹⁹, when dental plaque from the upper left deciduous molars was cultured for 48 h. Caries index is the number of smooth surface carious lesions detectable clinically or by means of X rays². a, Immunised; b, sham-immunised.

(± 1.8) SS cavities per animal in the immunised group. Only three fissure caries lesions appeared by 54 weeks in the deciduous teeth of two of the five controls and in none of the immunised monkeys.

The decrease in the incidence of dental caries is consistent with the results of previous immunisation studies¹, but there were two major differences in the experimental design. A formalin-killed *Strep. mutans* organism, isolated from a rhesus monkey, was used, unlike the heat-killed human strain of *Strep. mutans* in the previous studies. Furthermore, unlike the six immunising injections in the first study² and five injections in the second study¹, only two injections were used at an interval of 30 weeks. Indeed it is not certain that the boosting injection was necessary, as the IgG antibody titre at that stage had decreased by only one dilution step.

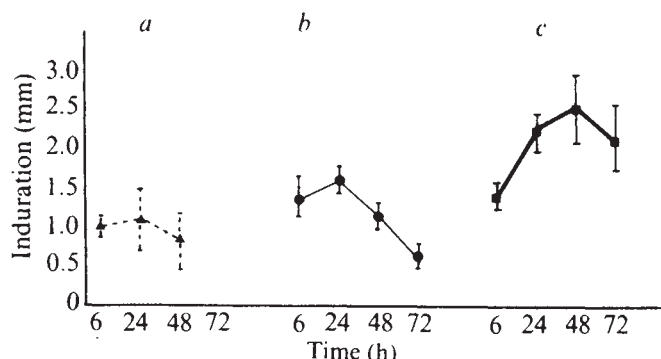


Fig. 2 Skin DH to *Strep. mutans*. A sample of 0.05 ml of Mickle disintegrated cells of *Strep. mutans* (10^9 cells per ml) or physiological saline was injected intradermally into the shaved abdominal wall of each animal. Skin induration was measured with calipers at the times stated and expressed as the mean specific increase in skin thickness ($\pm$ s.e.m.) due to *Strep. mutans*, after deduction of the skin thickness of the site injected with saline. *a*, Preimmunised; *b*, sham-immunised; *c*, immunised.

Cutaneous delayed hypersensitivity was measured 24 weeks after the second immunising injection (Fig. 2). The mean ($\pm$ s.e.) of skin induration of the three serial skin tests in each group of animals is given in Fig. 2 only for the broken cells, as the whole cells and culture extract failed to induce significant skin DH reactions. The results show a slight antigenic toxicity, in that the pre-immunised monkeys showed an induration of $1.0 (\pm 0.1)$ mm at 6 h (Fig. 2). The induration did not increase at 24 h, however, and decreased at 48 h. The sham-immunised monkeys showed a slight increase in induration from $1.42 (\pm 0.25)$ mm at 6 h to $1.62 (\pm 0.16)$ mm at 24 h and then a decrease at 48 and 72 h. In the immunised monkeys there was a significant increase from $1.42 (\pm 0.17)$ at 6 h to $2.23 (\pm 0.23)$ at 24 h ($P < 0.02$), to $2.54 (\pm 0.45)$ at 48 h ($P < 0.05$) and a decrease to $2.2 (\pm 0.45)$ at 72 h; the latter just failed to reach the 5% level of significance compared with the response at 6 h. The results indicate a skin DH reaction which reaches maximal value at 24–48 h.

When the lymphoproliferative response of the monkeys was examined, lymphocytes from preimmunised and sham-immunised monkeys failed to respond to any of the *Strep. mutans* preparations, so that these were combined into one control group (Table

Table 2 Lymphoproliferative responses of T and B lymphocytes

No.	T and B		Lymphocytes		B	
	c.p.m.*	SI†	c.p.m.	SI	c.p.m.	SI
1	1,331	12.8	1,390	1.8	524	1.2
2	1,468	2.6	427	3.1	841	1.7
3	2,158	4.5	1,004	3.6	3,551	1.1
4	2,573	9.9	1,484	7.0	nd	
5	3,194	4.2	2,959	4.2	nd	
6	3,264	7.1	1,339	9.7	nd	
7	1,291	2.1	1,819	3.4	nd	
8	1,716	3.4	1,514	4.2	nd	
	Mean	5.8		5.9		1.3
	s.e.	1.34		1.2		0.2

Lymphocyte transformation was assessed as for Table 1. T lymphocytes were prepared by passing lymphocytes over nylon wool adherence columns¹⁶. B lymphocytes were obtained by depleting lymphocytes of cells rosetting with neuraminidase-treated ovine erythrocytes¹⁷, by Ficoll-Triosil gradient sedimentation. Cell purity was assessed by direct staining for surface immunoglobulin with fluorescein-isothiocyanate-labelled polyvalent anti-human Ig (Wellcome).

*No antigen.

†*Strep. mutans* whole cell antigen.

1). Significant differences between controls and immunised monkeys were found with the culture extract ($P < 0.001$), cell walls ($P < 0.01$) and whole cells ($P < 0.02$) of *Strep. mutans*, respectively, though only the whole cells yielded moderate stimulation index (SI). The cellular responses were limited to T lymphocytes, for 96–98% purified T cells yielded significant stimulation by antigens, whereas 94% enriched B lymphocytes failed to respond (Table 2).

Thus both the lymphoproliferative response and skin DH to the immunising antigens were found 24 weeks after the last immunisation, with a good correlation between the two tests for the experimental and control groups. In contrast, phytohaemagglutinin (PHA) did not show a difference between the immunised and control monkeys, but significantly increased SI to pokeweed mitogen (PWM) were found in the immunised monkeys ($P < 0.02$; Table 1). Although the differential response to the mitogens might have been due to the adjuvant, this is unlikely, as a comparison has been made between animals injected with saline and Freund's incomplete adjuvant (unpublished). There was no significant difference in the lymphoproliferative responses between the saline and adjuvant injected monkeys with PHA (5.7 ± 1.7 and 8.3 ± 3.4) and with PWM (10.5 ± 5.6 and 16.6 ± 8.8) respectively.

As the T- and B-cell mitogen (PWM), unlike the T-cell mitogen alone (PHA), showed a greater proliferative response in the immunised animals, this argues in favour of a heightened B-cell responsiveness in the immunised animals. This is consistent with the high antibody response maintained throughout the experimental period.

Serum IgG and IgM antibodies were assayed by the indirect fluorescent antibody (FA) test (Fig. 1). IgG antibodies increased within 3 weeks of immunisation to a mean $\log_2$ titre of $5.3 (\pm 1.2)$ which increased to an optimum titre of $\log_2 8.3 (\pm 0.35)$ by week 9 and this level was maintained throughout the experimental period. IgM antibodies were also increased but the titre was lower than that of IgG throughout the experimental period (Fig. 1). The titre

Table 1 Lymphocyte transformation induced by antigens and mitogens

Group	No.	Mean c.p.m. ($\pm$ s.e.) of unstimulated cultures	Mean ($\pm$ s.e.) of SI to <i>Strep. mutans</i> antigens				Mitogens	
			Whole cells	Broken cells	Cell walls	Culture extract	PWM	PHA
Control	5	1,035 (± 357)	1.2 (± 0.2)	0.9 (± 0.2)	0.7 (± 0.1)	0.8 (± 0.2)	9.9 (± 3.9)	13.2 (± 5.6)
Immunised	3	863 (± 103)	5.9 (± 1.7)	2.2 (± 0.8)	2.0 (± 0.05)	2.7 (± 0.4)	33.8 (± 7.4)	12.2 (± 0.5)
<i>t</i>		-0.891	-3.623	1.941	8.785*	5.245	3.186	0.123
<i>P</i>		NS	<0.02	>0.05	<0.01	<0.001	<0.02	NS

1×10^6 Ficoll-Triosil separated lymphocytes, in 1.0 ml of 10% autologous serum in medium TC199 (Wellcome), were cultured for 120 h¹⁵. Antigens in previously determined optimal concentrations were added in 0.1-ml amounts. PHA (Wellcome) was used at 50 μ g per culture and PWM (Gibco) at 10 μ g per culture. Cells were labelled with 3 H-thymidine (1 μ Ci per 10^6 cells) for the last 18 h of culture and collected using an automatic sampling device. The c.p.m. of unstimulated cells are shown $\pm$ s.e.m. and SI is the ratio between antigen-stimulated and control cultures. NS, not significant.

*Only five monkeys tested.

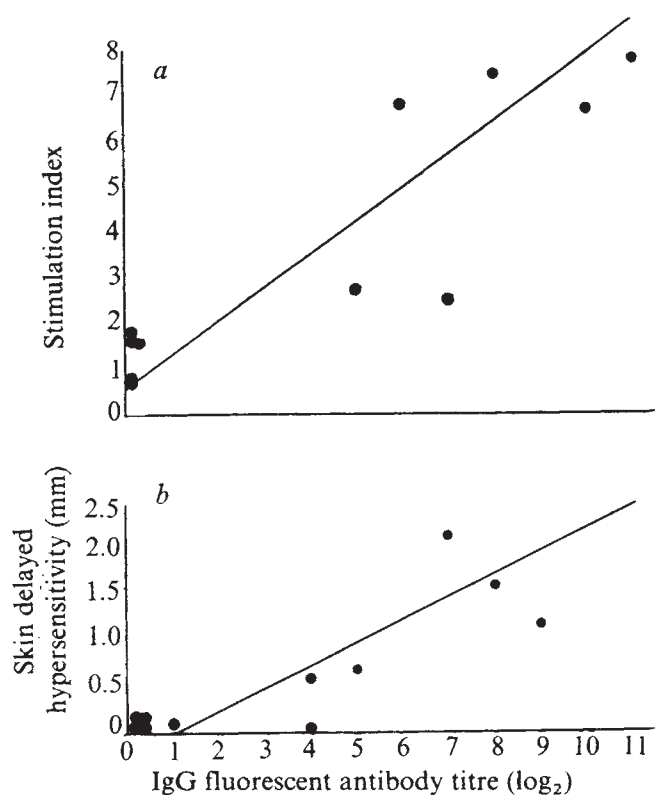


Fig. 3 Relationship between IgG antibodies to *Strep. mutans* and lymphocyte transformation (a) or skin DH (b). IgG fluorescent antibodies were assayed as described in Fig. 1. a, Lymphocyte transformation as in Table 1. SI is the ratio of ³H-thymidine uptake in *Strep. mutans* whole cell-stimulated and control cultures after 120 h of culture with 1×10^6 Ficoll-Triosil separated lymphocytes, in 10% autologous serum in medium TC199. b, Skin testing for DH as in Fig. 2. Results shown are mean specific increases in skin thickness 48 h after injection of *Strep. mutans* broken cells.

was maintained from weeks 3 to 30 at a level of about log₂ 3–4, but in spite of the second immunising injection at week 30, the titre fell to a level of less than log₂ 2 by week 45. None of the control sera showed IgG or IgM fluorescent antibodies.

The finding of significant skin DH as well as antibodies in immunised monkeys raised the question of whether the DH might be a measure of T-cell helper function⁹. To enable us to analyse linear regression with some confidence, three further monkeys previously immunised with *Strep. mutans* (group c) were included. A significant positive correlation ($r = 0.857$, $P < 0.001$) was found between the 48-h skin induration and the IgG fluorescent antibody titre (Fig. 3b) and this is consistent with a T-cell helper function in antibody formation. As with the skin DH reaction a significant positive correlation (Fig. 3a), was found between the proliferative response of lymphocytes and the IgG antibodies to *Strep. mutans* ($r = 0.884$, $P < 0.001$). IgM was neither correlated with the skin DH reaction ($r = 0.0826$, $P > 0.1$) nor with the lymphoproliferative response ($r = 0.411$, $P > 0.1$). The protective mechanism against dental caries may thus be dependent on the helper function of T lymphocytes in generating IgG antibodies, as has been found in other systems¹⁰.

As another measure of cellular responses, lymphokine production was assayed by the leukocyte migration inhibition test (LMIT)¹¹. The results failed to distinguish between the two groups, as both the immunised (three of three) and sham-immunised (two of three) groups yielded significant LMI tested with whole cells of *Strep. mutans* (Table 3). This finding raised the possibility that conversion of LMI in the control monkeys was induced by the overgrowth of *Strep. mutans* in the bacterial plaque, as two of three sham-immunised monkeys also gave a positive LMIT with broken cells of *Strep. mutans*. Indeed, the LMIT in preimmunised monkeys which had not been exposed to the human cariogenic diet and had little or no *Strep. mutans* was consistently negative, supporting the view that sensitisation to *Strep. mutans* may have been induced through the gingival crevicular epithelium or the gut, by the increased load of *Strep. mutans* in the bacterial plaque.

Sequential analysis of the CFU of *Strep. mutans* revealed consistently larger numbers of *Strep. mutans* in the control than in the immunised animals (Fig. 1). The results suggested that *Strep. mutans* were quantitatively related to the number of carious lesions in the control as well as the immunised groups, and that both the caries index and the number of *Strep. mutans* were decreased by immunisation.

It is also likely that not only the *Strep. mutans* antigen but also the adjuvant was required, as proposed by the modified "two signal model" in which an antigen-induced mitotic stimulus will result in the formation of a clone of antibody-forming cells only in the presence of a second, adjuvant stimulus¹². Indeed, negligible antibody titres and no lymphoproliferative responses were found on immunisation with *Strep. mutans* cells, without an adjuvant (unpublished).

In view of the great technical difficulties in monkeys of thymectomy *in vivo* or cell depletion *in vitro*, these have not been carried out. It is therefore not possible to draw definite conclusions as to T- and B-cell cooperation, though the results are consistent with the hypothesis that protection against *Strep. mutans* in dental caries involves T- and B-cell cooperation^{13,14}. This is based on finding: (1) a significant positive correlation between IgG antibodies and the proliferative response of lymphocytes to *Strep. mutans*, the latter being a function of T lymphocytes, and (2) a significant positive correlation between IgG antibodies and the skin DH reaction which may be a measure of T-cell helper function⁹.

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Table 3 Leukocyte migration inhibition test

Group	No.	No. of positive*, and (mean $\pm$ s.e.) of migration index to <i>Strep. mutans</i>		
		Whole cells	Broken cells	Culture extract
Sham-immunised	3	2 (81.3 $\pm$ 12.3)	2 (64.3 $\pm$ 19.9)	1 (79.3 $\pm$ 11.9)
Immunised	3	3 (74 $\pm$ 2.2)	0 (86.7 $\pm$ 2)	1 (90 $\pm$ 11.3)
Preimmunised	3	0 (105.7 $\pm$ 7.5)	0 (131.3 $\pm$ 2.3)	0 (140.7 $\pm$ 27.2)

Leukocytes were separated from peripheral blood using 3% dextran, washed twice and adjusted to 1×10^7 per ml in 10% horse serum with medium TC199. After filling, the capillary tubes (20 μ l) were plugged and centrifuged at 250g for 5 min., cut at the cell-fluid interface and placed in 1.0 ml of antigen-containing medium for 18 h in plastic chambers. Migration areas were recorded and measured by compensating planimetry. Migration index is the ratio between the area of leukocyte migration with and without antigen.

*Migration index < 80.

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Ly phenotype of T-cells releasing T-cell replacing factor

THE Ly phenotype of T cells which cooperate with B cells in the production of antibody has been described as Ly 1⁺2⁻ (refs 1 and 2). Helper T cells are distinct from cytotoxic T cells (Ly 1⁻ Ly 2⁺)^{1,2} and suppressor T cells (Ly 1⁻ 2⁺)^{3,4}. In tissue culture, helper activity does not depend on the presence of intact T cells but can be mediated by cell-free supernatants from stimulated T cells. Stimulation with antigen induces the release of antigen-specific helper factors⁵⁻⁶ as well as factors which support antibody response against unrelated antigens⁷. Factors similar or identical to the latter are produced by splenic T cells in response to alloantigens⁸⁻¹⁰ and concanavalin A¹¹. It is tacitly assumed that the mechanism by which helper T cells collaborate with B cells in the production of antibody is to be attributed to the release of these mediators. We report here that with respect to their Ly phenotypes, T cells which release T-cell replacing factor (TRF)⁸ in response to alloantigen belong to the same subpopulation of T cells as helper cells.

In the experiments summarised in Table 1, TRF was generated in a one-way allogeneic reaction: spleen cells from C57BL/6 (B6) mice were cocultured with spleen cells from (B6×DBA/2)F₁ mice. The subpopulation of T cells responsible for TRF release was determined by cytolysis of

the B6 spleen cells with antisera directed against distinct T-cell alloantigens. Factor activity was assayed by its ability to restore antibody formation against sheep red blood cells (SRBCs) in cultures of T-cell depleted spleen cells from (B6×DBA/2)F₁ mice.

The production of TRF was completely abrogated after treatment with anti-Ly 1 serum or anti-Thy 1 serum and complement. Treatment by anti-Ly 2 serum and complement had no effect. Mixture of anti-Ly 2-treated cells with anti-Ly 1 or anti-Thy 1-treated populations, containing half as many cells of each kind compared with the unmixed populations, produced about 50% of the TRF activity achieved with untreated or anti-Ly 2-treated populations.

These data show that the presence of Ly 1⁺2⁻ T cells is sufficient for TRF production. Ly 1⁺2⁻ or Ly 1⁺2⁺ cells neither produce TRF nor contribute to the production of TRF.

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Table 1 Effect of antisera on TRF production

B6 strain	Cytolysis with antiserum	TRF production	Assay for TRF	
		Ly phenotype of T cells tested for TRF production	BDF ₁ spleen cells	Anti-SRBC PFCs per culture (day 5)
B6 Ly 1.1 spleen	None	Ly 1 ⁺ 2 ⁻ ; Ly 1 ⁺ 2 ⁺ ; Ly 1 ⁻ 2 ⁺	T-cell depleted	550
(a)	Anti-Ly 1.1 + C	Ly 1 ⁺ 2 ⁺		0
(b)	Anti-Ly 2.2 + C	Ly 1 ⁺ 2 ⁻		670
(c)	Anti-Thy 1.2 + C	None		0
Equal mixture of (a) and (b)		Ly 1 ⁺ 2 ⁻ ; Ly 1 ⁻ 2 ⁺		200
Equal mixture of (b) and (c)		Ly 1 ⁺ 2 ⁻		324
B6 Ly 2.1 spleen	None	Ly 1 ⁺ 2 ⁻ ; Ly 1 ⁺ 2 ⁺ ; Ly 1 ⁻ 2 ⁺		770
	Anti-Ly 1.1 + C	"		700
	Anti-Ly 2.2 + C	"		640
	Anti-Thy 1.2 + C	None		0
No factors added			Untreated	5
				940

2×10^7 spleen cells per ml were suspended in 1 ml of 1:6 diluted anti-Ly 1.1 ((BALB/c×B6)F₁ anti-B6/Ly 1.1 thymocytes, absorbed with B6 thymocytes) or anti-Ly 2.2 ((C3H×B6/Ly 2.1)F₁ anti-B6 leukaemia ERLD, absorbed with B6/Ly 2.1 thymocytes) or 1:10 diluted anti-Thy 1.2 serum (A/Thy 1.1×AKR/H-2b)F₁ anti-A strain leukaemia ASL 1) or left untreated. After incubation for 30 min on ice, cells were washed with medium RPMI 1640 supplemented with 1% foetal calf serum. They were then resuspended at 1.5×10^7 cells per ml in 1:24 diluted selected rabbit complement and incubated for 40 min at 37 °C. Cells were washed twice in medium and resuspended in equal volumes of medium such that the density of the complement control was 1.0×10^7 cells per ml. To ensure serological specificity of anti-Ly antisera, the relevant congenic strain combinations were chosen. In the experimental group, spleen cells from the mouse strain B6/Ly 1.1 were treated with anti-Ly 1.1 or anti-Ly 2.2 antisera and complement. In control groups spleen cells of mice of the congenic strain B6/Ly 2.1 were treated with the same antisera and complement. In further control experiments all T cells were eliminated with anti-Thy-1.2 serum and complement. For the production of TRF⁸, equal volumes of B6 congenic cells (treated or untreated) and (B6×DBA/2)F₁ spleen cells were cultured in standard conditions¹¹. Supernatants were collected after 24 h, passed through 0.45-µm Millipore filters and stored at -70 °C. The helper effect was evaluated using T-cell depleted BDF₁ spleen cells sensitised with SRBC (basically following the Mishell-Dutton culture method¹¹). 0.2 ml of anti-Thy-1.2-treated BDF₁ spleen cells adjusted to a volume corresponding to 2×10^7 cells per ml in the complement control were added to multiwell plates (Falcon No. 3008), and 0.1-ml samples of the allogeneic supernatants described above or culture medium were added 24 h later. Plaque forming cells (PFCs) were determined by the Jerne haemolytic plaque assay after culture for 5 d. The data given are from one representative experiment. Repeat experiments gave similar results.

Role of chloride transport in regulation of intracellular pH

It is well known that introducing acid into a cell causes a rapid fall in intracellular pH (pH_i) which is followed by a slower rise¹⁻⁵. Since this return of pH_i towards its original value could not be accounted for by the passive transmembrane movement of H^+ , OH^- , or HCO_3^- , it was ascribed to the active removal of acid from the cell^{1,2}. In the squid giant axon, this acid extrusion is reversibly blocked by cyanide and greatly enhanced by HCO_3^-/CO_2 (ref. 3). More recently, it has been found that acid extrusion in the snail neurone also is stimulated by HCO_3^-/CO_2 (ref. 4), and that, in addition, it is inhibited by 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulphonic acid (SITS)⁵, a known inhibitor of anion fluxes in erythrocytes⁶. In this respect it is interesting to note that a component of Cl^- efflux in barnacle muscle also is stimulated by HCO_3^-/CO_2 and blocked by SITS⁷. We now report that acid extrusion in the squid axon requires internal Cl^- and ATP, that it is blocked by SITS, and that it is accompanied by the SITS-sensitive efflux of an equivalent amount of Cl^- . These observations suggest that acid extrusion actually involves the neutral exchange of external HCO_3^- for internal Cl^- .

Our experiments were carried out on giant axons of the squid *Loligo pealei*. To control the intracellular concentrations of low molecular weight solutes, we used the internal dialysis technique⁸: porous dialysis capillaries were introduced into cannulated axons, and flushed with solutions of known composition. In one series of experiments, pH_i was monitored by placing

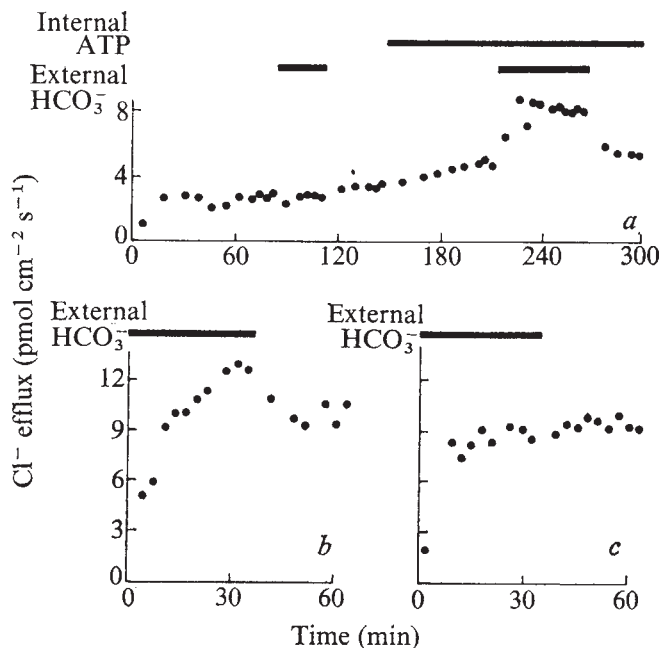


Fig. 2 $^{36}Cl^-$ efflux from internally dialysed squid axons. *a* ATP-dependence of HCO_3^- -stimulated Cl^- efflux. The axon was dialysed with a solution containing 150 mM Cl^- , and at zero time the flow of isotope-containing dialysis fluid was begun. The fluid superfusing the axon contained nominally 2 mM NaCN and was collected at 3- or 4-min intervals. An appropriate volume of a 2:1 toluene-Triton X-100 scintillation mixture was added¹⁰ and the samples counted long enough to achieve at least a 5% counting accuracy. There was no stimulation of $^{36}Cl^-$ efflux by 10 mM $HCO_3^-/0.4\%$ CO_2 unless the dialysis fluid contained ATP. V_m was -60 mV at the beginning of this experiment, and -53 mV at the end. *b* and *c*, Effect of 0.5 mM SITS on HCO_3^-/CO_2 -stimulated Cl^- efflux. In the experiment represented in *b* $^{36}Cl^-$ efflux fell when the HCO_3^-/CO_2 was removed from the ASW ($V_m = -60$ mV). In *c*, however, where the axon had been pretreated for 50 min with 0.5 mM SITS, removal of the HCO_3^-/CO_2 had no effect ($V_m = -64$ mV). In four axons, 0.5 mM SITS caused the HCO_3^-/CO_2 -stimulated Cl^- efflux to decline from 10.1 ± 1.5 to 7.2 ± 1.0 pmol cm⁻² s⁻¹. pH of dialysis solution 6.8; temperature 15 °C.

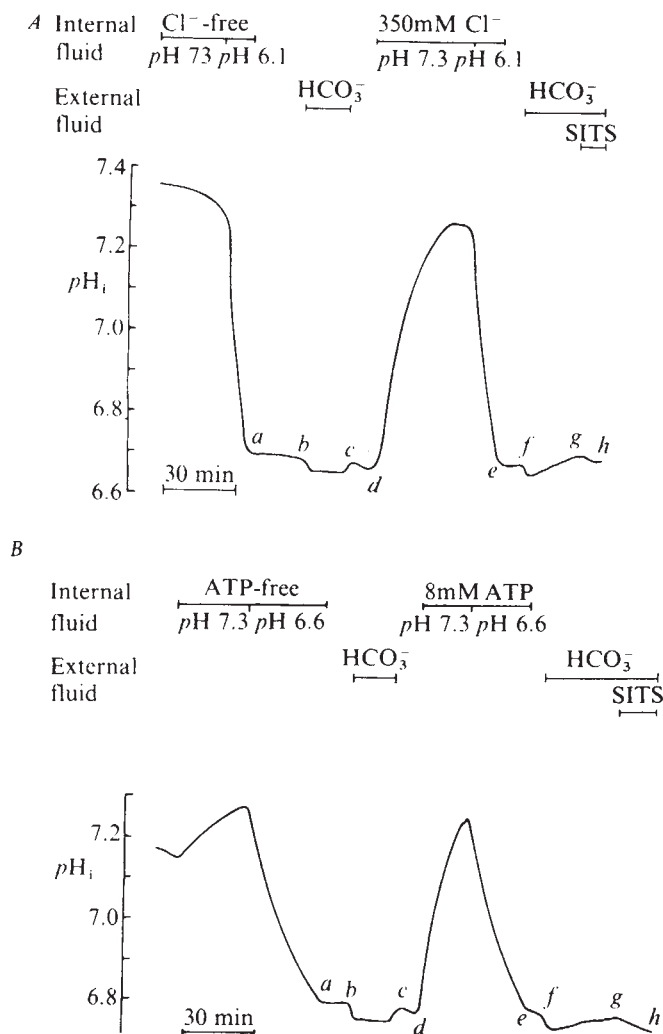


Fig. 1 *A*, Effect of $(Cl^-)_i$ on the acid extrusion. The axon was dialysed first with Cl^- -free (glutamate replacing Cl^-) and then with 350 mM Cl^- fluid, while pH_i was monitored with a glass electrode. In the absence of internal Cl^- , HCO_3^-/CO_2 (segment *bc*) did not stimulate acid extrusion ($n = 17$), whereas in the presence of 350 or 150 mM Cl^- (not shown), HCO_3^-/CO_2 (*fg*) did stimulate acid extrusion ($n = 14$). SITS 0.5 mM (*gh*) blocked the HCO_3^- -stimulated acid extrusion ($n = 7$). Membrane potential (V_m) was -57 mV at the outset of this experiment, and -47 mV at the end. The period of dialysis was always at least 40 min, a length of time sufficient to attain isotopic equilibration with $^{36}Cl^-$ (see Fig. 2). In the low Cl^- ASW, NaCl and KCl were replaced by sodium isethionate and $MgCl_2$ by $MgSO_4$. *B*, ATP dependence of acid extrusion. The axon, pretreated for 1 h in nominally 2 mM NaCN ASW, was dialysed first with an ATP-free and then with an 8 mM ATP solution (150 mM Cl^-). Although HCO_3^-/CO_2 failed to stimulate acid extrusion in the absence of internal ATP (*bc*), HCO_3^-/CO_2 did stimulate this extrusion when ATP (*fg*) was present ($n = 3$). The acidification during *ef*, which probably reflects breakdown of the exogenous ATP, was minimised by pretreating the axon with 10^{-8} M ouabain and by including oligomycin ($12 \mu g ml^{-1}$) in the dialysis fluid. In this experiment, the initial and final V_m was -61 mV. Temperature of both experiments 22 °C. The pH of the 10 mM $HCO_3^-/0.4\%$ CO_2 ASW was 8.0; all other ASWs in this study had a pH of 7.7. Unless otherwise noted, the composition of the dialysis fluid was (mM): 150 KCl, 200 K glutamate, 50 Na glutamate, 4 $MgSO_4$, 190 taurine, 0.5 EGTA, 10 HEPES and 0.5 phenol red.

pH-sensitive and reference microelectrodes into the axon² alongside the dialysis capillary. In the other series, Cl^- efflux was measured in axons dialysed with solutions containing $^{36}\text{Cl}^-$.

The dependence of acid extrusion on internal Cl^- was investigated by measuring this extrusion, both in the presence and absence of internal Cl^- . The axon of Fig. 1A was superfused with low (20 mM) Cl^- seawater, and was first dialysed with a Cl^- -free solution (pH 7.3). Acid was then introduced into the axon by lowering the pH of the dialysis fluid to 6.1. When the pH_i had fallen to 6.7 (point *a*), the flow of dialysis fluid was halted so that the subsequent course of pH_i was determined by cellular processes. In a normal axon, this fall in pH_i (acid challenge) would be followed by a rise, the rate of this alkalisation being an index of acid extrusion. In this axon depleted of internal Cl^- , however, pH_i continued to fall (segment *ab*). Moreover, $\text{HCO}_3^-/\text{CO}_2$ failed to reverse this decline (*bc*). In contrast, when Cl^- was reintroduced into the axon by dialysing with a 350 mM Cl^- solution, and the pH_i was once more reduced to 6.7 (*e*), HCO_3^- -stimulated acid extrusion was observed (*ef*). Dialysis with 150 mM Cl^- gave identical results (not shown). Treating the axon with 0.5 mM SITS (*gh*) blocked the HCO_3^- -stimulated acid extrusion.

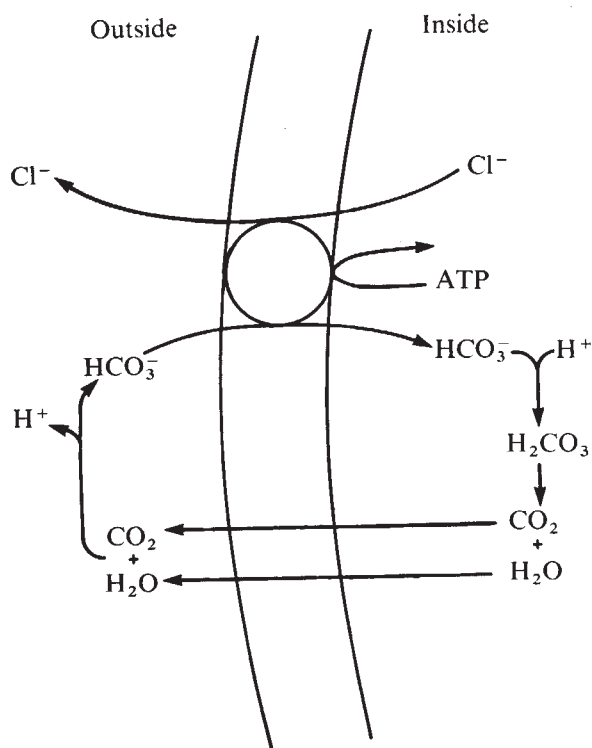


Fig. 3 Proposed scheme for acid extrusion. An ATP-dependent mechanism would inject external HCO_3^- in exchange for internal Cl^- . Once inside, the HCO_3^- would combine with H^+ to produce $\text{CO}_2 + \text{H}_2\text{O}$, which would then leave the cell and yield $\text{H}^+ + \text{HCO}_3^-$ once again. The net effect is to eject HCl from the cell.

The previously described inhibition of acid extrusion by cyanide³ suggested that acid extrusion might require ATP. One can reduce internal ATP to very low levels by dialysing with an ATP-free solution in the presence of cyanide⁹. In this manner, we lowered $(\text{ATP})_i$ in the axon of Fig. 1B, and then challenged the axon with acid. No alkalisation (that is, no acid extrusion) was observed when dialysis was halted (*a*) even in the presence of $\text{HCO}_3^-/\text{CO}_2$ (*bc*). When the axon was dialysed with an ATP-containing solution (*d*) in the continued presence of cyanide and then challenged with acid, the pH_i continued to fall (*ef*) after cessation of dialysis (possibly due to breakdown of exogenous ATP). But this decline was reversed when $\text{HCO}_3^-/\text{CO}_2$ was added (*fg*). SITS again blocked this acid extrusion (*gh*), causing pH_i to fall at about the same rate as in segment *ef*.

The preceding experiments show that internal Cl^- and ATP are required for HCO_3^- -stimulated, SITS-sensitive acid extrusion. The evidence that externally applied $\text{HCO}_3^-/\text{CO}_2$ enhances Cl^- efflux from barnacle muscle, and that this HCO_3^- -stimulated efflux is similarly SITS sensitive⁷ prompted us to consider the possibility that Cl^- efflux is linked to acid extrusion. We found that at relatively high pH_i , adding 10 mM $\text{HCO}_3^-/0.4\% \text{CO}_2$ had very little effect on acid extrusion. Similarly, we found that in these conditions (pH_i 7.3), 10 mM $\text{HCO}_3^-/0.4\% \text{CO}_2$ does not significantly increase Cl^- efflux. When the pH_i was lowered to about 6.7 by dialysis, however, both acid extrusion (Fig. 1) and Cl^- efflux were found to be enhanced by $\text{HCO}_3^-/\text{CO}_2$: in 12 experiments 10 mM $\text{HCO}_3^-/0.4\% \text{CO}_2$ increased Cl^- efflux from 7.7 ± 0.6 (s.e.m.) to 11.6 ± 1.0 $\text{pmol cm}^{-2} \text{s}^{-1}$ (15°C). The response of Cl^- efflux and acid extrusion to removal of ATP provides another point of comparison between the two processes. As was the case with acid extrusion (Fig. 1B), Cl^- efflux did not respond to $\text{HCO}_3^-/\text{CO}_2$ when the axon was deprived of ATP (Fig. 2a); responsiveness was restored when ATP was added to the dialysis fluid. In addition, just as with acid extrusion, $\text{HCO}_3^-/\text{CO}_2$ stimulation of Cl^- efflux was inhibited by SITS, as can be seen by comparing Fig. 2b and c.

Thus, both HCO_3^- -stimulated acid extrusion and Cl^- efflux require ATP and are blocked by SITS. In three acid extrusion experiments in which the temperature was the same as in the Cl^- studies (15°C), HCO_3^- -stimulated acid extrusion was estimated to be 4.8 ± 0.9 $\text{pmol cm}^{-2} \text{s}^{-1}$, rather close to the HCO_3^- -stimulated Cl^- efflux (3.9 $\text{pmol cm}^{-2} \text{s}^{-1}$). It is attractive to hypothesise (Fig. 3) that the squid axon possesses an ATP-dependent pump which responds to acid challenges by taking up HCO_3^- from the external solution and extruding an equivalent amount of Cl^- . Since the entering HCO_3^- is converted to CO_2 and H_2O , both of which subsequently leave the axon, the net effect of this transport mechanism is to extrude HCl .

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Acetylcholine receptor degradation measured by pulse chase labelling

THERE is increasing evidence that the turnover of the cholinergic receptor protein in developing or adult muscle fibres varies in the course of synaptogenesis or as a consequence of denervation^{1,2}. Muscle activity is involved in this regulation³, but the mechanisms of the control processes and the chemical signals which mediate these effects remain largely unknown.

To investigate this question several methods have been developed for the measurement of acetylcholine-receptor (AChR) turnover in embryonic muscle in tissue culture⁴ and denervated or neonatal rat diaphragm⁵. We present

here quantitative methods for the direct measurement of AChR turnover which depend on the incorporation of the radioactive precursor ^{35}S -L-methionine into newly synthesised receptor polypeptides and subsequently purification of radioactive receptor by affinity chromatography (ref. 4 and manuscript in preparation). These techniques have been applied to the determination of receptor half life in primary tissue cultures of foetal calf skeletal muscle. Results reported here indicate that AChR polypeptides with toxin binding activity have a half life of about 17 h. This value is identical to that obtained indirectly by measurement of the degradation of ^{125}I - α -bungarotoxin (^{125}I -BuTx) bound to receptor sites.

To measure directly the degradation of AChR, cultures of dissociated foetal calf skeletal muscle cells were pulse labelled with ^{35}S -L-methionine (10 Ci mmol^{-1} , CEA, France) for 7 h, 90 h after establishment of the cultures^{4,5}. After washing, the cells were chased with myoblast conditioned medium supplemented with $5 \times 10^{-5}\text{ M}$ L-methionine. At different times after the chase, 10 culture dishes were washed with warmed phosphate buffer saline (PBS) and stored at -80°C . AChR was purified from labelled cells by techniques described previously⁴ with the modifications indicated in the legend to Table 1.

Table 1 is a summary of AChR purifications from two pulse chase experiments. In every case, receptor activity (as determined by ^{125}I -BuTx binding assay⁹) from the labelled extract was quantitatively adsorbed to the affinity support of Erabutoxin-b coupled to Sepharose 4B (data not shown). The yield of each purification was therefore dependent on the efficiency of specific elution by the cholinergic agonist decamethonium. As can be seen in column C, Table 1, the yield or % recovery of toxin binding sites from different purifications varied from 11 to 29%. The observed variability is attributed to variation in specific elution from the affinity support. Recent experiments have shown that the receptor not recovered by decamethonium elution remains bound to the affinity support, suggesting it is not recovered in an inactive form.

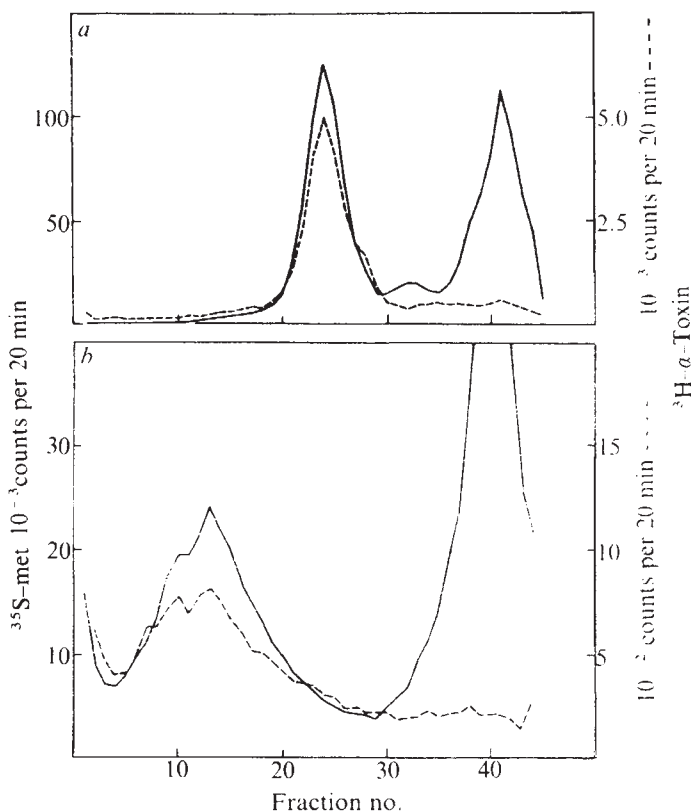


Fig. 1. Column purified receptor activity, after removal of decamethonium on DE-52 cellulose, was incubated at 4°C with ^3H - α -toxin from *N. nigricolis*, and normal or anti-*E. electricus* acetylcholine receptor rabbit serum. These incubations were centrifuged in isokinetic 10–40% sucrose gradients for 24 h at 40,000 r.p.m. in Beckman SW 41 rotor^{11,12}. Fractions were collected from the bottom of the tube and counted in Triton containing scintillation cocktail¹² for ^3H (solid line) and ^{35}S (dashed line).

Table 1 AChR purification from pulse chase labelled cells.

Hours after chase	A pmol ^{125}I -BuTx sites adsorbed	B pmol ^{125}I -BuTx sites recovered	C ^{125}I -BuTx sites % recovery	D Total ^{35}S -met c.p.m.	E AChR ^{35}S -met c.p.m.	F Corrected AChR ^{35}S -met c.p.m.
Experiment 1						
0	30	3.5	11.4	5.48×10^8	4.07×10^4	3.58×10^5
14	34	9.9	29.1	4.25	5.40	1.85
23	34	8.9	26.1	3.80	2.99	1.15
Experiment 2						
0	30	5.9	19.3	4.45	1.37	7.1×10^4
16.5	39	5.9	15.0	3.54	0.63	4.2
28	26	5.9	22.7	3.10	0.61	2.7
40	24	5.9	25.0	2.42	0.30	1.2

Cells were labelled as described in the text. Frozen cells were scraped from the plates in distilled water. The water extracts were centrifuged at 100,000g for 30 min. The membrane pellet was suspended in 1% Triton X-100 in 0.18 M NaCl, 0.025 M NaH_2PO_4 pH 7.6 containing 10^{-4} M phenylmethylsulphonyl fluoride, 10^{-4} M benzethonium chloride and 10^{-3} M EDTA to inhibit protease activity¹⁴. After extraction for 2 h at 4°C , the extract was centrifuged at 100,000g for 30 min. The supernatant was removed and filtered through a plug of glass wool. Triton extracts were adsorbed to an excess of affinity support, consisting of Erabutoxin b, coupled to cyanogen bromide, activated Sepharose 4B ($100\text{ }\mu\text{g ml}^{-1}$)^{15,16}. Adsorption was complete ($>80\%$) in 3–5 h. After the complete adsorption of receptor activity from the ^{35}S -labelled extracts, the affinity columns were incubated with an excess of cold crude extract containing receptor. Receptor adsorption was monitored by the ^{125}I -BuTx binding assay. Column A is the amount of total toxin-binding activity (^{35}S labelled plus cold) bound to the column. The columns were washed with 0.36 M NaCl, 0.05 M NaH_2PO_4 pH 7.6, 1% Triton, until the level of ^{35}S radioactivity in the wash was reduced to background. Toxin binding activity was released from the column by incubation with 0.1 M decamethonium at 4°C for 40 h. After dilution, the toxin column eluates were adsorbed to and eluted from DE-52 cellulose columns to remove decamethonium. The toxin-binding activity was then assayed by ^{125}I -BuTx binding. The yield of each purification was calculated from the toxin-binding activity recovered relative to that initially bound, column C. Total ^{35}S -methionine c.p.m., column D, was determined by hot TCA precipitation of an aliquot of the crude water extract. The amount of AChR ^{35}S -methionine c.p.m. was determined by analysis of the toxin column purified material (after DE-52 chromatography) on sucrose gradients. Complete recovery of input ^{35}S c.p.m. was obtained. Only the 9S toxin binding peak was summed and recorded in column E. Finally, these values were corrected to 100% recovery, column F.

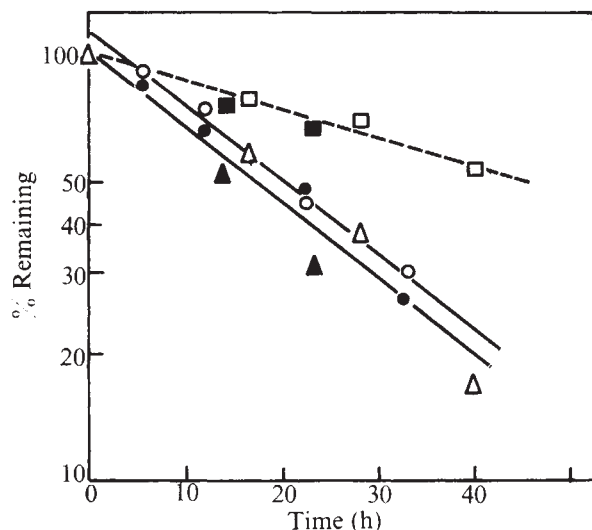


Fig. 2 AChR degradation. The corrected values for AChR ^{35}S -c.p.m. remaining after the chase from Table 1 were normalised to a zero time 100% figure and plotted here as a first-order decay as open and filled triangles for the two separate experiments. The total protein ^{35}S -c.p.m. was similarly normalised and plotted as open and closed squares. The loss of bound ^{125}I -BuTx was studied as described by Devreotes and Fambrough⁸. Cells were incubated with ^{125}I -BuTx (prepared as described by Vogel *et al.*, 1972 (ref. 13) overnight at a concentration of 5×10^{-9} M. Unbound toxin was washed free by six successive washes with warmed complete culture medium. Finally, cells were returned to the culture with complete myoblast conditioned medium. At different times, four labelled plates were washed several times with PBS and scraped in 1% Triton X-100, 0.18 M NaCl, 0.025 M NaH_2PO_4 (pH 7.6). These extracts were counted directly in a gamma counter. All values from two independent experiments were normalised to a zero time of 100% and were plotted as open and closed circles.

At each step of the purification, the ^{35}S -methionine content of the sample was determined by scintillation counting. Column D, Table 1, lists the total hot trichloroacetic acid precipitable material. In column E is recorded the summed ^{35}S -methionine radioactivity of the affinity purified receptor which was observed to cosediment with the 9S peak of toxin-binding activity in a 10–40% isokinetic sucrose gradient^{1,2}. The fraction of ^{35}S -c.p.m. thus sedimenting represented 63–83% of the total in the gradient and was quantitatively shifted to a more rapidly sedimenting form by preincubation with rabbit antiserum prepared against purified AChR from *Electrophorus electricus*. One example of such an analysis is shown in Fig. 1 (ref. 4). Furthermore, the affinity purified material was found to be of high purity by two-dimensional high resolution gel electrophoresis (ref. 7 and J.P.M., manuscript in preparation).

The values obtained by sucrose gradient analysis were finally corrected for variation in the yield and are recorded in column F. It can be calculated that the synthesis of ACh receptor makes up only a very small fraction of global protein synthesis, about 0.065% in experiment 1 and 0.016% in experiment 2.

To calculate a receptor half life from the experimental data in Table 1, the values in column F were normalised to a zero time figure of 100%. In a similar fashion, a mean half time was calculated for total protein values in column D. The respective half lives were 17 h for the AChR and 48 h for total protein.

The value of 17 h for ACh-receptor half life by direct pulse chase experiments is in good agreement with values previously published for AChR from chick skeletal myotubes in cultures^{1,8} and extra synaptic receptor of denervated rat diaphragm^{2,9}. All of these previous measurements have been made by the indirect method in which the

degradation of bound ^{125}I -BuTx is observed. We have confirmed the half-life measurements by the indirect method in our tissue culture system and have found a value of 17 h, identical to that determined by the pulse chase experiments. This agreement lends support to the argument that the toxin-binding method is a valid probe of receptor metabolism^{8,9}.

From studies of loss of bound toxin to denervated or embryonic rat diaphragm, two rates of receptor degradation have been suggested^{9,10}. One, extrasynaptic, with a relatively high rate of turnover having a $T_{1/2}$ of 8–22 h, and a second more stable, synaptic, species with $T_{1/2} > 100$ h. The reason for this considerable difference in degradation rate is not known. Furthermore, there is indirect evidence that the activity dependent alteration of extrasynaptic receptor levels is regulated at the level of polypeptide synthesis^{11,16}. Quantitative studies of receptor synthesis making use of the technique applied here to receptor degradation should permit the elucidation of the control mechanisms involved in the steady-state level of ACh receptor. It will be possible to determine how nerve-induced muscle activity functions to signal alterations in macromolecular synthesis, at what level (transcriptional or translational) is receptor synthesis controlled and what are the precursor-product relationships between synaptic and extrasynaptic receptor.

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Anticholinergic and membrane activities of amantadine in neuromuscular transmission

AMANTADINE hydrochloride (1-adamantanamine hydrochloride, Symmetrel) is an antiviral agent which prevents certain viruses from penetrating cells^{1–3}. In addition, this drug is also effective in relieving clinical symptoms shown in parkinsonism^{4–6}; this action of amantadine may depend on its capacity to increase the synthesis and release of dopamine from dopaminergic cells of the basal ganglia^{9,10}. To obtain additional evidence which might aid in explaining

these remarkably diverse effects of amantadine, we used single-cell electrophysiological techniques to study its action on junctional and extrajunctional membranes. Our results show that amantadine, in clinically effective concentrations, rapidly inhibits neuromuscular transmission, and when applied over a longer period of time the drug also exerts a substantial effect on the conductile membranes of muscle fibres.

In the first series of experiments we studied the action of amantadine on the indirectly-stimulated nerve-muscle preparation. Frog (*Rana pipiens*) sartorius muscle-sciatic nerve preparations were bathed in HEPES-buffered Ringer solution (112.4 mM Na⁺, 2.5 mM K⁺, 1.8 mM Ca²⁺, 117.1 mM Cl⁻, 3.0 mM HEPES buffer, pH 7.4) bubbled with 100% oxygen and containing dextrose (1 g l⁻¹). The sciatic nerve was stimulated electrically (0.1 ms min⁻¹) at a level 2.5 times that required for initiating the maximal isometric tension output. When the tension output had become constant, the preparation was curarised in two stages to produce a stable partial neuromuscular block¹¹. The nerve and muscle were first soaked for 10 min in HEPES-buffered Ringer solution containing 1.4 μ M *d*-tubocurarine (*d*TC) after which the *d*TC concentration was reduced to 0.7 μ M for the remainder of the experiment. Amantadine was added to the bath only after muscle tension output in the *d*TC-treated preparation had been stable for at least 60 min. For experiments where resting potentials (r.p.s), action potentials (a.p.s), endplate potentials (e.p.s) and miniature endplate potentials (m.e.p.s) were recorded intracellularly, the cutaneous pectoris nerve-muscle preparation was used *in vitro*. The preparation was bathed in HEPES-buffer Ringer solution, and where e.p.s were recorded, the two-stage curarisation technique described above was used. All experiments were done at room temperature (19–25 °C).

The results of isometric tension output measurements in partially curarised muscle are shown in Fig. 1. It can be seen that application of amantadine produced a simple reduction tension output which was reversed when this drug was removed. In three additional experiments, amantadine was applied to non-curarised nerve-muscle preparations. At 800 μ M amantadine, the tension output was reduced to 35% of control after 40 min exposure to the drug. At 500 μ M amantadine, the tension output fell to 60% of control after 55 min of drug application. Amantadine at 150 μ M did not produce a reduction in muscle tension output.

In the next series of experiments, e.p.s were recorded intracellularly from single muscle fibres of the curarised cutaneous pectoris muscle while nerve stimuli were applied once every 10 s. After a 30-s control period, for which three e.p.s were recorded and averaged, the junctional region was microperfused with bathing solution containing either 50 or 150 μ M amantadine. As shown in Fig. 2, the e.p.s were immediately and progressively reduced in

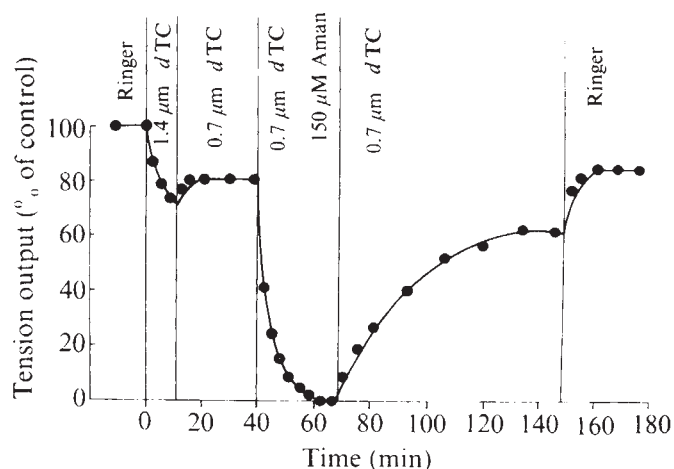


Fig. 1 Effect of amantadine on the isometric tension output of partially curarised, indirectly-stimulated sartorius muscle-sciatic nerve preparation of frog. Average of three muscles. Aman, amantadine.

amplitude following application of amantadine. During the 8-min period following cessation of amantadine application, the e.p.p. amplitude rose but did not reach the control value. The incomplete recovery is partially the result of a 10–12-mV fall in r.p. which occurred during the recording because the muscle twitch was not completely blocked in these experiments. More complete recovery would probably have occurred if additional time had been allowed, and if the muscle had been washed with amantadine-free bathing solution.

In the next group of experiments we showed that the slow reduction of resting potential observed in the previous experiments is not produced as a result of a postjunctional depolarising action of amantadine. The nerve-muscle preparation was treated as before with HEPES-Ringer solution plus *d*TC (two stages) but in this case only one e.p.p. was recorded from each individual muscle fibre of the sample group. Thereafter the muscle was bathed in HEPES-Ringer solution containing *d*TC plus amantadine (50 or 150 μ M) and a single e.p.p. was recorded from an additional group of individual fibres over a period of the next 30 min. The results of such experiments are summarised in Table 1. They show that there is a rapid reduction in the amplitude of the e.p.p. to 40% of control during the first 5 min of exposure to 50 μ M amantadine, and a reduction to 10% of control follows 5 min of exposure to 150 μ M amantadine. It can be seen that amantadine progressively depresses the amplitude of the e.p.p. during the entire 30-min period of its application, and it is also evident that amantadine does not depolarise the postjunctional membrane under these conditions.

Table 1 Effect of amantadine on resting potential and endplate potentials of partially curarised frog muscle fibres

No. of muscles	Amantadine (μ M)	Duration of soaking (min)	r.p. (mV)	n	e.p.p. (mV)	n
3	Control	—	91.8 $\pm$ 0.37*	49	11.9 $\pm$ 0.52*	49
	50 μ M	1–5	90.0 $\pm$ 0.67	14	4.7 $\pm$ 0.74	14
	50 μ M	5–10	90.5 $\pm$ 1.04	9	2.8 $\pm$ 0.74	9
	50 μ M	10–20	92.0 $\pm$ 1.36	8	1.7 $\pm$ 0.60	8
	50 μ M	20–30	91.3 $\pm$ 1.37	13	1.2 $\pm$ 0.20	13
2	Control	—	91.5 $\pm$ 0.44	28	10.2 $\pm$ 0.82	28
	150 μ M	1–5	93.0 $\pm$ 1.06	9	0.95 $\pm$ 0.26	9
	150 μ M	5–10	92.2 $\pm$ 0.67	10	0.66 $\pm$ 0.19	10
	150 μ M	10–20	92.0 $\pm$ 0.49	13	0.70 $\pm$ 0.17	13
	150 μ M	20–30	94.0 $\pm$ 0.77	8	0.38 $\pm$ 0.14	8

*. Standard error of mean.

n, Number of fibres.

The local perfusion technique was also employed to observe the effects of amantadine on the amplitude and frequency of m.e.p.p.s. Microperfusion of two endplates with Ringer solution containing 300 μ M amantadine reduced the m.e.p.p. amplitude to zero in 12–48 s and the amplitude recovered to 70–85% of control 7 min after amantadine application had ceased. With 150 μ M amantadine, the m.e.p.p. amplitude in three endplates fell to 40–55% of control in 1 min and recovery was 65–90% complete in 5–7 min. At 50 μ M amantadine the m.e.p.p. amplitude in two endplates was reduced to 50% in 2 min and recovered to 70–80% in 4–7 min. The time course for depression and recovery of m.e.p.p. amplitude observed in these experiments was similar to that shown by e.p.p.s in the previous experiments. Amantadine at 50 and 150 μ M seemed to reduce the m.e.p.p. frequency but this limited observation needs verification, partly because amantadine reduces m.e.p.p. amplitude and thus the frequency count may be inaccurate because some low amplitude m.e.p.p.s may have gone undetected in the baseline noise of our recordings.

We have so far provided evidence that amantadine at 50 and 150 μ M has significant blocking action at the frog neuromuscular junction since it causes reduction in the tension output of the indirectly stimulated, partially curarised nerve-muscle preparation, and it also substantially reduces the amplitudes of e.p.p.s and m.e.p.p.s. The reduction in the amplitudes of m.e.p.p.s and e.p.p.s may be interpreted on the basis that amantadine reduces the sensitivity of the postjunctional membrane to acetylcholine and the following experiments were carried out to test this supposition. Local perfusion of nine junctions (three muscles) with 100 μ M amantadine reduced the m.e.p.p. amplitude from a control value of 0.35 mV to 0.11 mV in ~ 25 s. The m.e.p.p. frequency (min^{-1}) fell from 37 (control) to 15. In seven of these perfused junctions which were tested for response to nerve stimulation, five showed block of neuromuscular transmission (average e.p.p. amplitude 18 mV). During iontophoretic application of 5-ms pulses of carbamylcholine to the postjunctional membrane, microperfusion with 100 μ M amantadine reduced the carb depolarisations from 2.1 mV (control) to 0.3 mV in ~ 70 s (nine fibres). Thus the reduction in postjunctional sensitivity produced by amantadine is sufficient to account for its rapid action in reducing the amplitude of m.e.p.p.s and e.p.p.s and hence in causing a block in neuromuscular transmission.

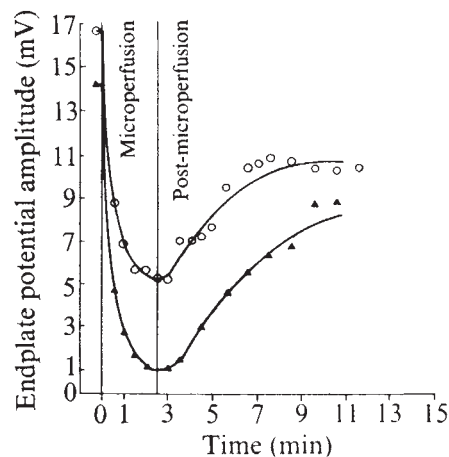


Fig. 2 Effect of amantadine on the amplitudes of endplate potentials recorded from partially curarised muscle fibres. ○, average of five endplates, 50 μ M amantadine; ▲, average of six endplates, 150 μ M amantadine.

In the final series of experiments, we investigated the direct action of amantadine on the muscle fibre conductile membrane. No change in r.p. or a.p. was produced during the first 30 min of application of 150 μ M amantadine. After 1 h of exposure to 150 μ M amantadine, however, depolarisation of the muscle fibres was evident and thereafter the depolarisation increased steadily with time. From Table 2, it may be seen that the average reduction of the resting potential was 8 mV between the first and second hour, and 18 mV during the second to third hour of exposure to 150 μ M amantadine. In addition, the amplitude, rate of rise and rate of fall of the a.p. were significantly reduced by application of 150 μ M amantadine for these time periods.

The results of our study indicate that amantadine rapidly inhibits neuromuscular transmission by reducing the response of the muscle postjunctional membrane to acetylcholine. When applied for relatively prolonged periods, it affects the resting and action potentials of extrajunctional excitable membranes. If amantadine produces similar changes in the resting and action potentials of the pre-synaptic neuronal membrane, one might predict that this drug would reduce the quantal output per nerve impulse. Unfortunately, it is not feasible to test this speculation by using the usual experimental methods because amantadine strongly inhibits postjunctional cholinergic receptors.

Table 2 Effect of 150 μ M amantadine on junctional and extrajunctional resting potentials and action potentials of frog muscle fibres

Conditions	n	r.p. (mV)	a.p. (mV)	m.r.r. (V s^{-1})	m.r.f. (V s^{-1})	Duration† (ms)	e.p.p.r.r. (V s^{-1})
Extrajunctional							
Ringer	12	91.7 $\pm$ 0.41*	126.0 $\pm$ 0.94*	505.0 $\pm$ 12.40*	134.1 $\pm$ 2.28*	0.80 $\pm$ 0.008*	—
Amantadine	11	83.2 $\pm$ 0.81	117.6 $\pm$ 1.09	419.0 $\pm$ 15.08	80.9 $\pm$ 2.84	1.05 $\pm$ 0.01	—
1st–2nd hour							
Amantadine	11	73.3 $\pm$ 1.71	102.0 $\pm$ 3.85	306.0 $\pm$ 35.78	71.8 $\pm$ 4.22	1.00 $\pm$ 0.02	—
2nd–3rd hour							
Return to Ringer	12	87.0 $\pm$ 0.85	123.0 $\pm$ 1.29	465.4 $\pm$ 15.51	117.5 $\pm$ 2.78	0.88 $\pm$ 0.01	—
15–45 min							
Junctional							
Ringer	6	89.3 $\pm$ 1.42	119.0 $\pm$ 2.03	648.3 $\pm$ 31.56	81.6 $\pm$ 5.42	0.80 $\pm$ 0.04	191.6 $\pm$ 15.79
Amantadine	12	79.6 $\pm$ 2.08	110.8 $\pm$ 3.19	530.0 $\pm$ 37.63	54.5 $\pm$ 5.54	1.10 $\pm$ 0.01	65.3 $\pm$ 8.66
1st–3rd hour							

*, Standard error of mean.

r.p., Resting potential.

n, Number of records.

†, Duration of a.p., measured at 0 mV line.

m.r.r., Maximal rate of rise of a.p.

a.p., Action potential.

m.r.f., Maximal rate of fall of a.p.

e.p.p.r.r., Maximal rate of rise of endplate potential.

We suggest that the beneficial effect of amantadine in parkinsonism may depend to some extent on the capacity of this drug to inhibit cholinergic transmission in certain neuronal circuits involved in the central control of muscular movement. The demonstrated action of amantadine on extrajunctional membranes may provide a route whereby the mode of action of this drug in preventing penetration of cells by viruses may ultimately be understood.

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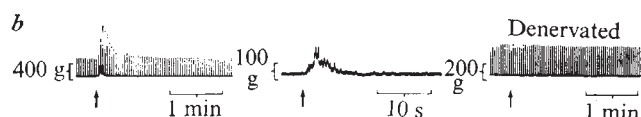
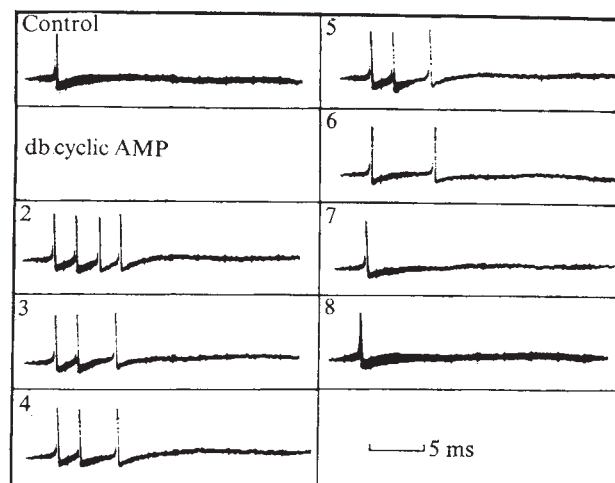


Fig. 1 *a*, SBR produced in a soleus motor axon by db cyclic AMP. The first trace shows the response to a single stimulus applied before the drug. Subsequent traces show the responses to similar stimuli applied once every 2.5 s after the administration of db cyclic AMP ($100 \mu\text{g kg}^{-1}$). The time mark represents 5 ms. *b*, Effects of db cyclic AMP on the force of contraction of the soleus muscle. The left record represents the response produced by administration of the agent (arrow, $100 \mu\text{g kg}^{-1}$ intra-arterial) to a neurally stimulated muscle. The middle record represents the response to db cyclic AMP (arrow, $200 \mu\text{g kg}^{-1}$ intra-arterial) of an unstimulated muscle. The right record represents the effect of the agent (arrow, $200 \mu\text{g kg}^{-1}$ intra-arterial) on a chronically denervated muscle.

Evidence for a prejunctional role of cyclic nucleotides in neuromuscular transmission

THERE is evidence for¹⁻³ and against^{4,5} the involvement of cyclic nucleotides in the release of neurotransmitters. We have investigated this question using a method more suited to the detection of prejunctional drug actions than those used previously. Instead of trying to infer the action of nucleotides on nerve endings by recording endplate potentials or muscle contractions *in vitro*, we recorded from single motor axons of cat soleus nerves *in vivo*. We found that dibutyl (db) cyclic AMP initiated activity in unstimulated motor axons and produced stimulus-bound AMP, db cyclic GMP, sodium butyrate and 5' AMP had no repetitive activity (SBR) in stimulated axons, whereas cyclic effect. NaF and theophylline also initiated activity in unstimulated axons and produced SBR in stimulated axons. Pretreatment with the theophylline potentiated the effects of db cyclic AMP or NaF. The results suggest that cyclic AMP is involved in the excitation-secretion sequence of mammalian motor nerve endings.

The preparations have been described before⁶. Cats were anaesthetised with α -chloralose (70 mg kg^{-1}), the popliteal fossa was dissected and all branches of the tibial nerve except that to the soleus muscle were cut. Similarly, all branches of the popliteal artery except the posterior tibial were occluded. Both heads of the gastrocnemius muscle were removed and the tendon of the soleus muscle was attached to a strain gauge. Dorsal laminectomy (L4-S1) exposed the ventral root of spinal nerve L7, which was severed close to the cord and its distal stump divided until a strand containing a single active axon to the soleus nerve was found. The strand was placed across bipolar platinum recording electrodes. A bipolar platinum stimulating electrode was placed on the soleus nerve and a

supramaximal pulse lasting 0.1 ms was applied every 2.5 s.

Denervated muscles were prepared by anaesthetising cats with intramuscular ketamine (25 mg kg^{-1}) and aseptically cutting the sciatic nerve at the sciatic notch. Two weeks later the animal was anaesthetised with intravenous chloralose (70 mg kg^{-1}) and the leg was prepared and mounted as before. A pair of electrodes was formed by sewing 33-gauge stainless steel wire in two series of concentric loops at the tendinous end of the muscle. A supramaximal pulse lasting 2 ms was applied every 2.5 s.

Seven reagents were used. The first three, *N*⁶-2'-*O*-dibutyl-adenosine-3',5'-monophosphoric acid, cyclic (db cyclic AMP), *N*²-2'-*O*-dibutyl-guanosine-3',5'-monophosphoric acid, cyclic (db cyclic GMP) and adenosine-3',5'-monophosphoric acid, cyclic (cyclic AMP) are nucleotide derivatives. Of these, the first two cross cell membranes easily, while the last does not. The fourth, NaF, is an activator of adenylate cyclase⁷, the fifth, theophylline, is an inhibitor of phosphodiesterase and the last two, sodium butyrate and 5' adenosine monophosphate (5'AMP), are hydrolysis products of db cyclic AMP. Each compound was dissolved in 0.85% NaCl solution so that 0.1 ml contained the amount to be administered per kg, and 0.1 ml per kg of the solution was injected into the popliteal artery.

Dibutyl cyclic AMP in doses ranging from 50 to $200 \mu\text{g kg}^{-1}$ caused SBR in motor axons (Fig. 1), with maximum intensity occurring after the first stimulus after the injection—less than 2.5 s after injection. Repetitive potentials were of high frequency, 300-400 Hz, but the train after each stimulus was always less than 12 ms. Neural activity was transmitted to the muscle where it caused repetitive activation and a brief tetanic contraction stronger than the simple twitch of the control period. This

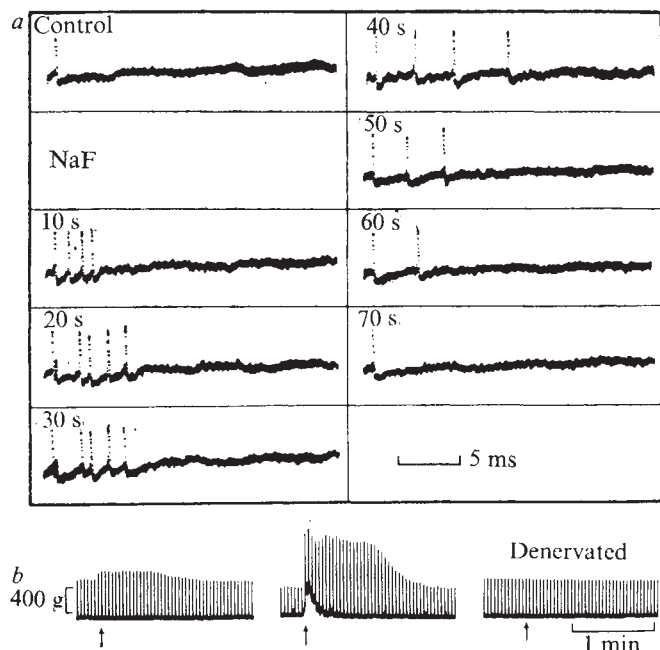


Fig. 2 *a*, SBR produced in a soleus motor axon by NaF. The first trace shows the response to a single stimulus applied before the drug. Subsequent traces show the responses to similar stimuli applied at the given time intervals after the intra-arterial administration of NaF ($200 \mu\text{g kg}^{-1}$). Time mark represents 5 ms. *b*, Effects of intra-arterial NaF (arrows) on the force of contraction of the soleus muscle. The first two records represent the effects of 50 and $200 \mu\text{g kg}^{-1}$, respectively, on a neurally stimulated preparation. The third trace represents the effect of $400 \mu\text{g kg}^{-1}$ on a chronically denervated muscle.

is shown in the mechanograms of Fig. 1, which also shows that the intensity, duration and time course of the repetitive activity were related to the dose of db cyclic AMP. Larger doses of db cyclic AMP ($> 100 \mu\text{g kg}^{-1}$) also initiated activity in unstimulated preparations. Immediately after injection, bursts of action potentials appeared in the nerve and the muscle underwent a series of rapid asynchronous contractions, causing the baseline of the record to rise (Fig. 1). The nerve activity consisted of brief bursts of between one and 150 action potentials which had a frequency of 300–500 pulses per s. Modally, the drug-initiated activity (DIA) consisted of 10–25 potentials and was complete within 3 s of injection. Dibutyl cyclic AMP in doses up to $800 \mu\text{g kg}^{-1}$ had no effect on denervated muscle (Fig. 1).

Cyclic AMP in doses up to $800 \mu\text{g kg}^{-1}$ or sodium butyrate or 5'AMP in doses up to 1 mg kg^{-1} had no effect on innervated or denervated preparations. Dibutyl cyclic GMP in doses of $1\text{--}2,000 \mu\text{g kg}^{-1}$ had no effect on innervated or denervated preparations and when given before or after the injection of db cyclic AMP, it did not affect the response to the latter nucleotide.

NaF produced responses which were qualitatively the same as those of db cyclic AMP (Fig. 2), causing SBR and DIA in motor axons, and potentiating the strength of indirectly evoked muscle contractions and causing asynchronous muscle contractions in unstimulated preparations. The threshold dose was $50 \mu\text{g kg}^{-1}$ and a ceiling of effect occurred at $400 \mu\text{g kg}^{-1}$. Injections of NaF up to $800 \mu\text{g kg}^{-1}$ had no effect on denervated muscle.

Theophylline caused SBR in motor axons and potentiation of muscle contraction strength (Fig. 3). The threshold dose was about $100 \mu\text{g kg}^{-1}$ and maximal effect occurred with doses of about $600 \mu\text{g kg}^{-1}$. The effect did not occur as rapidly as after db cyclic AMP or NaF; in most cases

5–10 s elapsed. The repetitive potentials were low in frequency, below 300 Hz, and the trains lasted up to 25 ms (Fig. 3).

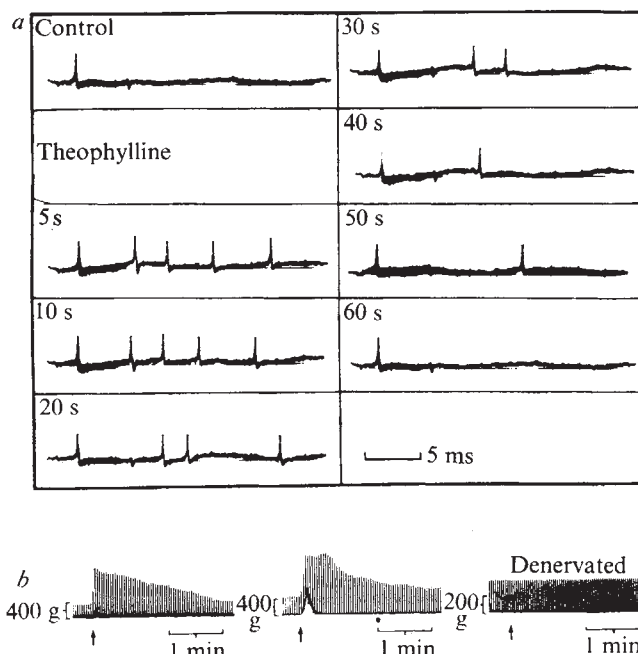
Theophylline also produced DIA in nerves and muscles but the dose required was larger than that needed to produce SBR. The activity differed from that initiated by db cyclic AMP and NaF in that there was a delay of several seconds between the injection of the drug and the appearance of the first potentials, and DIA was absent or much less intense if the stimulator was turned off for several minutes before injection. Theophylline had no effect on chronically denervated muscle.

Pretreatment with doses of theophylline below threshold for overt effects (such as $50 \mu\text{g kg}^{-1}$) lowered the threshold dose of db cyclic AMP or NaF by a factor of about 5. Theophylline also greatly increased the intensity and duration of all effects of db cyclic AMP or NaF.

There are several possible mechanisms responsible for SBR in motor axons but prolongation of the afternegativity of an action potential evoked in the nerve terminal is essential to them all^{8,9}. The prolonged afternegativity re-excites a faster recovering proximal segment of the axon (for example, the last node of Ranvier) and thereby produces SBR. Thus, in our experimental conditions, the production of SBR in the axon and/or potentiated contractions in its muscles shows that a reagent reacted with the nerve ending to prolong its afternegativity.

The lack of effect of each compound on chronically denervated muscle suggests that they do not cause a significant direct depolarisation of the post-junctional cell. This is substantiated by the reports that the compounds did not affect muscle membrane potential or amplitude of miniature end plate potentials (m.e.p.p.s) of nerve-muscle preparations. In the absence of significant

Fig. 3 *a*, SBR produced in a soleus motor axon by theophylline. The first trace shows the response to a single stimulus applied before the drug. Subsequent traces show the responses to similar stimuli applied at the given time intervals after intra-arterial administration of theophylline $100 \mu\text{g kg}^{-1}$. Time mark represents 5 ms. *b*, Effects of intra-arterial theophylline (arrows) on the force of contraction of the soleus muscle. The first two records represent the effects of 100 and $400 \mu\text{g kg}^{-1}$, respectively, on a neurally stimulated preparation. The third record represents the effect of $400 \mu\text{g kg}^{-1}$ on chronically denervated muscle.



postjunctional depolarisation, the most likely explanation for the production of DIA is that db cyclic AMP or NaF caused the nerve ending to become sufficiently depolarised to trigger action potentials in the axon and to release transmitter. Theophylline similarly seemed to cause depolarisation of nerve endings, but seemed to require prior activation of the nerve.

As cyclic AMP had no effect in our preparation and as its most significant difference from db cyclic AMP is in the capacity to penetrate cells, our results suggest that dibutyl cyclic AMP acts intracellularly to cause depolarisation of the nerve membrane. Dibutyl cyclic GMP, which also enters cells, also had no effect, suggesting that the effect is not a nonspecific response to intracellular nucleotides. Cyclic nucleotides have been shown to facilitate the influx of Na and/or Ca into cells¹⁰⁻¹⁴, and this can carry depolarising currents and trigger the release of transmitter¹⁵. These observations thus suggest that an increase in the influx of Na and/or Ca could have been the basis for the effects of dibutyl cyclic AMP in our experiments.

The similarity of the effects of NaF, an activator of adenylate cyclase, and dibutyl cyclic AMP, suggests that this enzyme, an important component of cyclic nucleotide systems, is present in motor nerve endings.

Because theophylline induces SBR in motor axons and potentiates and prolongs the effects of db cyclic AMP, phosphodiesterase is likely to be present in the nerve ending. The potentiation and prolongation of the effects of NaF by theophylline suggests that phosphodiesterase participates in the destruction of cyclic nucleotide formed after activation of adenylate cyclase in nerve endings.

If a cyclic nucleotide system was a link between the nerve action potential and transmitter release, it should have four characteristics. First, it should be quiescent until it is activated. Second, it should normally be activated by a nerve action potential. Third, it should be turned on, run its course and be turned off in a few ms. Fourth, it should be modified by exogenous cyclic nucleotides and reagents that affect the enzymes of the cyclic nucleotide system. Our results are in accord with these expectations and suggest mechanisms by which cyclic AMP might participate in neuromuscular transmission.

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Release and metabolism of substance P in rat hypothalamus

THE isolation and characterisation of the undecapeptide substance P from extracts of central nervous tissue has promoted renewed interest in the function of this compound in the central nervous system (CNS)¹. In addition, the development of a sensitive and specific radioimmunoassay² and complementary immunohistochemical techniques³ has provided evidence for a differential distribution of substance P in the CNS^{4,5}. Application of substance P to the spinal cord of neonatal rats has been shown to produce a potent depolarising action on motoneurons⁶. Elsewhere in the CNS iontophoretic application of substance P elicits a characteristic neuronal excitation which although slow in onset is long lasting⁷⁻¹⁰. If substance P is to be considered as a neurotransmitter in the CNS, demonstration of its release from nerve terminals in response to physiological depolarisation is the next essential prerequisite¹¹. We have therefore studied the release of substance P from rat hypothalamus, and have

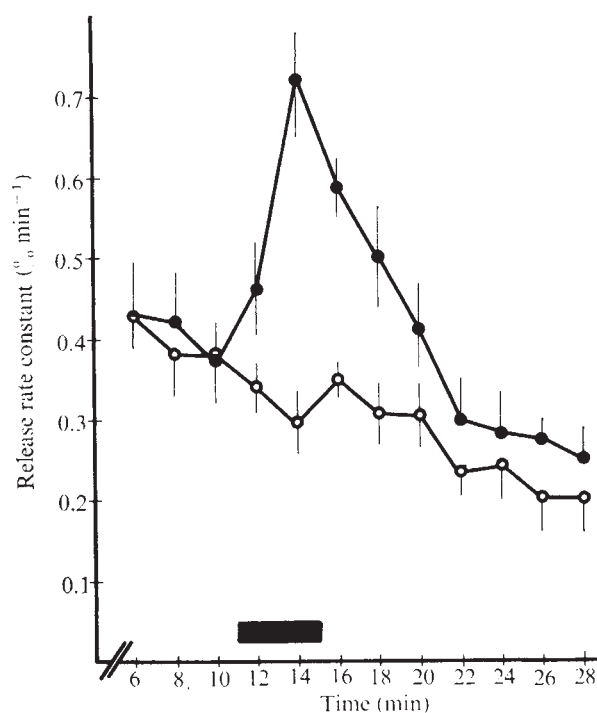


Fig. 1 Release of substance P-like immunoreactivity from rat hypothalamus slices. Adult male Wistar rats (200-250 g) were killed by decapitation, the brains were removed immediately and the hypothalamus was dissected using a modification of the method of Glowinski and Iversen²² (mean wet weight: 45.5 ± 2.1 mg; $n = 10$). Two hypothalami were used in each experiment, chopped in two directions at 0.2-mm intervals and transferred to a Perspex perfusion chamber. The slices were then superfused at 37 °C with Krebs-bicarbonate solution with glucose and containing 0.5% bovine serum albumin at a rate of 200 μ l min⁻¹. The superfusate was collected at 2-min intervals and stored on ice. Twelve minutes after the onset of perfusion, the potassium concentration of the medium was raised to 47 mM for 4 min (horizontal bar). In some experiments, the calcium concentration of the superfusion medium was reduced to 0.1 mM and the magnesium concentration raised to 3.5 mM. Samples of 200 μ l of serial superfusate fractions were removed for determination of substance P-like immunoreactivity by radioimmunoassay. Hypothalamic slices recovered after the superfusion were extracted and substance P-like immunoreactivity is expressed as a fractional rate constant, which represents the amount released per minute in each sample as a percentage of the total tissue content of peptide. Each point is the mean $\pm$ s.e.m. for five experiments. ● Normal medium; ○, low Ca²⁺ medium.

demonstrated a potassium-evoked and calcium-dependent release of immunoreactive material. In addition preliminary results suggest that metabolic degradation rather than tissue uptake is responsible for the inactivation of substance P after its release from brain tissue.

Throughout this study substance P was measured by a radioimmunoassay, essentially similar to published methods². The lower limit of sensitivity was approximately 10 pg of substance P per assay sample. Antibody specificity was checked against the related peptide eledoisin (Sigma), which failed to cross react with substance P even when present in 1,000-fold molar excess.

Using this radioimmunoassay procedure, we have found that rat hypothalamus contains a relatively high concentration of substance P-like immunoreactivity (SPLI)¹², in agreement with previous results in human brain². Rat hypothalamus was, therefore, chosen as a suitable tissue for release studies². A selective, calcium-dependent release of neurotransmitter candidates both *in vivo* and *in vitro* can be induced by raising the potassium concentration in the release studies². A selective, calcium-dependent release of neurotransmitter candidates both *in vivo* and *in vitro* can be induced by raising the potassium concentration in the external medium^{13,14}. Accordingly, to study the release of SPLI, rat hypothalamic slices were continuously superfused with Krebs-bicarbonate solution as described previously¹⁵, and potassium-evoked release was monitored. In

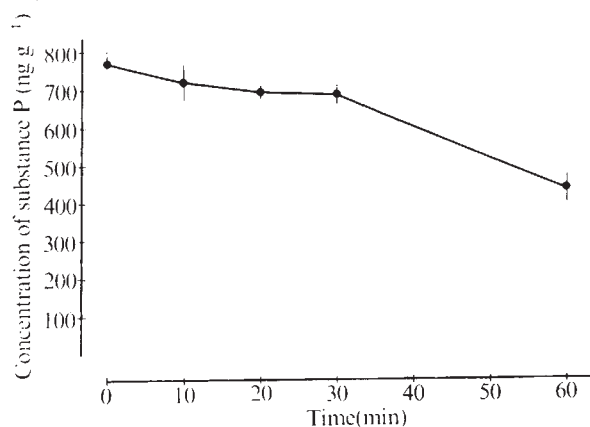


Fig. 2 Metabolism of substance P in rat hypothalamic slices. Portions (10 mg) of a suspension of rat hypothalamic slices were incubated in 1 ml of Krebs-bicarbonate solution with added glucose containing 0.5% bovine serum albumin at 37 °C for various times. At the end of incubation, samples were stored on ice and centrifuged at 1,500g for 10 min, and both supernatant and tissue were assayed for substance P-like immunoreactivity. Each point is the mean $\pm$ s.e.m. for at least six determinations.

preliminary experiments the recovery of internal standards of substance P added to tissue extracts or superfusate samples was more than 94%. To prevent absorption of substance P to the apparatus and collection tubes, bovine serum albumin (0.5% w/v) was routinely added to the superfusion medium. Elevation of the potassium concentration in the superfusing medium to 47 mM for 4 min resulted in a marked increase in the rate of release of SPLI (Fig. 1). The maximum increase in release occurred within 4 min of potassium application, and efflux returned to spontaneous levels 6 min after restoration of normal potassium concentrations. Superfusion of hypothalamic slices with a medium in which the calcium concentration had been reduced to 0.1 mM and the magnesium concentration raised to 3.5 mM did not significantly affect the spontaneous efflux.

The marked increase in release evoked by 47 mM potassium, however, was completely abolished in the low Ca^{2+} and high Mg^{2+} medium. Potassium-evoked release of SPLI from rat hypothalamic tissue therefore seems to

be highly calcium dependent, as is the case with other neurotransmitter release mechanisms. SPLI remaining in the tissue at the end of each release experiment was determined. After 30 min of superfusion with normal Ca^{2+} medium, including 4 min of potassium stimulation, hypothalamic slices contained $663 \pm 50 \text{ ng g}^{-1}$ (mean $\pm$ s.e., $n = 5$), a value not significantly different from that in freshly prepared slices not subjected to superfusion ($745 \pm 40 \text{ ng g}^{-1}$; $n = 6$) or in slices superfused with the low Ca^{2+} /high Mg^{2+} medium for 30 min ($637 \pm 86 \text{ ng g}^{-1}$; $n = 5$). The mean rate of spontaneous efflux of SPLI in normal or modified medium was equivalent to a total release of 8–10% of the tissue content during 30 min. The apparently small pool of tissue substance P available for release, and the high degree of retention of the peptide in the tissue, are similar to the results obtained with other transmitter substances in this type of release experiment^{14,15}.

If substance P is released as a neurotransmitter, mechanisms for inactivating the free peptide might be expected to exist in nervous tissue. To examine this possibility, we incubated hypothalamic slices in Krebs-bicarbonate medium at 37 °C for various times up to 60 min, without superfusion. Figure 2 shows that the rate of decline of SPLI in the hypothalamic tissue was slow: levels after 30 min were 89% of those at the onset of incubation. This figure is in good agreement with the values found in release experiments. In the absence of superfusion, however, no free peptide was detectable in the incubation medium at any time. After 60 min of incubation it was still not possible to detect SPLI in the incubation medium, although at this time tissue levels had fallen to 57% of the original values. It seems possible therefore that although tissue stores of substance P are reasonably stable, liberated substance P is susceptible to degradation within the hypothalamic tissue. Incubation of synthetic substance P with rat brain homogenates has been shown to result in rapid inactivation, with the release of all constituent amino acids¹⁶. In the release studies reported above, the continuous superfusion procedure presumably reduced such inactivation.

The possibility that the released peptide might be inactivated by a tissue uptake mechanism was also investigated in the rat cerebral cortex, dorsal and ventral spinal cord, substantia nigra, hypothalamus and cerebellum. Slices of these tissues (0.2 mm $\times$ 0.2 mm) were incubated with ^{125}I -N¹-tyrosyl-substance P in the concentration range 10^{-7} – 10^{-5} M at 0, 25 and 37 °C. In no case did tissue:medium ratios significantly exceed unity, even with incubations of up to 40 min duration. Incubation of rat hypothalamic slices with unlabelled synthetic substance P at the same concentrations also failed to demonstrate any accumulation of the peptide. It seems unlikely, therefore, that substance P is actively accumulated by the tissue from which it is released.

Our results demonstrate that endogenous SPLI can be released from rat hypothalamic tissue in response to a depolarising stimulus by a calcium-dependent mechanism. Although the cellular origin of the released peptide is not known, it seems likely that release occurs from substance P-containing nerve terminals in the hypothalamus. Previous biochemical studies have shown that most of the substance P in homogenates of brain¹⁷ and hypothalamus¹⁸ is localised in synaptic vesicle particles. Furthermore, immunohistochemical findings suggest that most of the SPLI in rat hypothalamus is concentrated in nerve terminals⁴. Our results also agree with those of earlier studies which showed the presence of substance P-like activity in the superfusates collected from spinal cord¹⁹ and cerebral cortex²⁰, although these studies were performed before the advent of the specific and sensitive radioimmunoassay procedure. There

have also been preliminary reports of a stimulus-evoked release of SPLI from rat spinal cord^{11,21}.

The above demonstration of release adds further weight to the hypothesis that substance P serves a neurotransmitter role in the nervous system. Although more information is needed on possible mechanisms of inactivation, our results suggest that tissue metabolism rather than a reuptake mechanism is the important pathway for terminating the actions of the released peptide.

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was added to protect substance P against oxidation. Dithiothreitol at this concentration did not affect spinal reflexes, which indicated that the drug did not interfere with the oxygenation of the spinal cord. In most experiments, either 7 nM pepstatin⁵ or trasylol (0.5 U ml⁻¹) was added to the perfusion fluid to inhibit the degradation of substance P by proteases. Four to six lumbar dorsal roots were placed in suction electrodes for electrical stimulation. One of the ventral roots (L3–L5) was placed in another suction electrode with a tip diameter just fitting the size of the ventral root for extracellular recording, and the reflex responses were monitored during the stimulation periods. The perfusion rate was 0.2–0.6 ml min⁻¹, and after the preparation had been washed for at least 1 h, the perfusate was collected for 15 or 30 min into test tubes which each contained 1 ml of 60 mM dithiothreitol. Samples were frozen immediately, lyophilised and submitted to radioimmunoassay for substance P (ref. 6). The details of the assay system are described elsewhere⁷. Antisera against substance P were raised in rabbits, using synthetic substance P coupled with human α -globulin. ¹²⁵I-N⁶-tyrosyl-substance P was used as tracer. The minimum detectable amount of substance P by the assay system was 2.5–5 pg per incubate. The antibody cross reacted with some of the shorter C-terminal analogues of substance P (deca-, nona- and octapeptides) but not with other shorter C-terminal analogues.

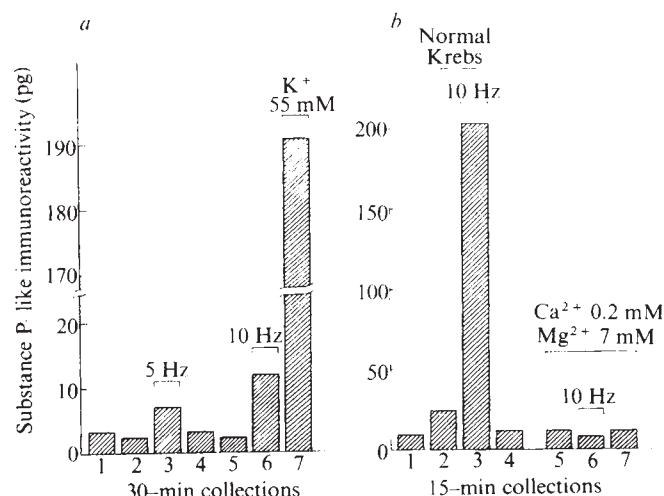


Fig. 1 Substance P-like immunoreactivity in the perfusates of isolated spinal cords of newborn rats. Each column represents the immunoreactivity expressed as an amount of substance P in a sample of about 7 ml. Stimulation periods and frequencies are indicated in the figure. The duration of each stimulating pulse was 0.01 ms in (a), and 0.3 ms in (b). In the experiment shown in (b), the preparation was first perfused with normal Krebs solution and then with a Ca²⁺-deficient and Mg²⁺-rich Krebs solution.

Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat

EVIDENCE is accumulating that substance P (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂)¹ is an excitatory transmitter released from primary afferent nerve terminals in mammalian spinal cord (for reviews see refs 2 and 3). An important criterion for identification of a transmitter is the demonstration of release of the candidate substance from the nerve terminals in response to presynaptic stimulation. We report here the release of substance P-like immunoreactivity from isolated rat spinal cord into the bathing fluid during stimulation of dorsal roots.

Spinal cords from the thoracic level to the caudal end of 1–2-d-old Wistar rats were isolated, hemisected sagittally and placed in a 0.3-ml bath⁴. The preparation was perfused with oxygenated Krebs solution at 27 °C to which 6 μ M dithiothreitol

Figure 1 shows the results of two typical experiments. In the experiment shown in Fig. 1a, rectangular pulses of 0.01 ms were used for stimulation. During the resting periods, the amounts of substance P-like immunoreactivity in 30-min collection samples were below the limit of accurate measurement. Stimulation of the dorsal roots at a frequency of 5 Hz induced a slight increase, and stimulation at 10 Hz caused a definite increase in the release of substance P-like immunoreactivity. When the preparation was perfused with modified Krebs solution in which K⁺ concentration was increased to 55 mM, a large amount of the immunoreactivity was released into the perfusate. In the experiment illustrated in Fig. 1b, longer stimulation pulses (0.3 ms) were used to activate the smaller fibres in the dorsal roots. During the first stimulation period in normal Krebs solution a remarkable increase of substance P-like immunoreactivity in the perfusate was found, and this was associated with continual reflex responses throughout the stimulation period. But when the preparation was perfused with

modified Krebs solution containing 0.2 mM Ca^{2+} and 7 mM Mg^{2+} , which abolished monosynaptic and polysynaptic reflexes, the same stimulation produced no change in the immunoreactivity in the perfusate. Similar results were obtained when the experimental procedures were reversed, that is another spinal cord was first perfused with the Ca^{2+} -deficient and Mg^{2+} -rich Krebs solution, where the stimulation at 10 Hz for 15 min produced no reflex responses and no significant change of the immunoreactivity in the perfusate; the preparation was then perfused with normal Krebs solution and the same stimulation produced continual reflex responses and a marked increase in substance P-like immunoreactivity.

The results presented here demonstrate the calcium-dependent release of substance P-like immunoreactivity from the isolated rat spinal cord in response to stimulation of dorsal roots. Angelucci⁸ has shown that a substance P-like agent is released from perfused frog's spinal cord, but obtained no clear evidence as to whether its rate of release increased during reflex activity. Jessell *et al.*⁹ demonstrated the potassium-evoked and calcium-dependent release of substance P-like immunoreactivity from rat hypothalamus. The substance P-like immunoreactivity collected in our experiments is likely to have originated from nerve terminals of primary afferent fibres in the spinal cord, because previous studies^{10,11} gave good evidence for the occurrence of substance P in these fibres. Thus our findings support the view that substance P is an excitatory transmitter released from dorsal root fibres.

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Macromolecules, steroid binding and testosterone secretion by rabbit testes

THE formation of testosterone by the testis has been studied extensively and the general characteristics of the process have been described¹. But the mechanism by which testosterone escapes from the steroidogenic cell and then from the testis is largely unknown². Eik-Nes³ speculated that testosterone secretion could be explained by the presence of plasma constituents which bind the hormone. These plasma constituents might include erythrocytes, albumin and sex-hormone-binding globulin which have association constants for testosterone of 1×10^3 (ref. 4), 3×10^4 (ref. 5) and 1×10^9 (ref. 6), respectively. On the basis of this information, we hypothesised that testosterone secretion is dependent on diffusion from the steroidogenic cell into the blood and that this diffusion is enhanced by the presence of steroid-binding macromolecules in the blood. We report here a test of this hypothesis.

Rabbit testes were perfused *in vitro*^{7–9} at 10 ml per g of testis per h with medium containing macromolecules

which differed widely in steroid-binding capacity. These initial studies focused on serum albumin for three reasons: (1) its physical, chemical, and steroid-binding characteristics are well known; (2) it is the only steroid-binding protein commercially available in a highly purified but stable form, and (3) its plasma concentration and high capacity for steroids make it a likely candidate for one component of a macromolecular steroid sink in blood.

The medium for control perfusions consisted of Krebs–Ringer bicarbonate buffer, pH 7.4, 3% (w/v) bovine serum albumin (BSA), fraction V (Reheis Chemical Co.), washed bovine erythrocytes (25% in the medium), crystalline penicillin ($1,000 \text{ IU ml}^{-1}$), glucose (100 mg%) and a saturating concentration of NIH-LH-S₁₇, ovine (100 ng ml^{-1}). The medium was gassed with a mixture of air and 5% carbon dioxide. For experimental perfusions, fraction V BSA was omitted and in some cases replaced with 3% 4× crystalline BSA (ICN Pharmaceuticals), 3% ovalbumin (Sigma) or 3% Dextran T70 (Pharmacia). Venous effluent from perfused testes was collected at hourly intervals during each experiment. The blood cells were sedimented by centrifugation and washed with an equal volume of 0.9% (w/v) saline. The saline was combined with the plasma portion of the venous effluent and frozen. The testosterone content of this material or of testicular homogenate was measured by gas–liquid chromatography¹⁰.

In vitro binding of testosterone to BSA, ovalbumin and Dextran was determined by equilibrium dialysis, according to Westphal's method¹¹. After dialysis for 18 h at 4 °C, testosterone ($0.5 \mu\text{g ml}^{-1}$) was found to be 86% bound to BSA, whereas it was only 22% bound to ovalbumin and less than 5% bound to Dextran.

In a further series of experiments, one testis from each of 24 rabbits was perfused with medium containing 3% fraction V BSA. The contralateral testes from six of the rabbits were perfused with medium without albumin; six with medium containing 4× crystalline albumin; six with medium containing 3% Dextran, and six with medium containing 3% ovalbumin. This experimental design enabled us to compare testosterone secretion by paired testes, thus eliminating the large variance in secretion between animals. Testosterone content of the venous effluent collected during the second hour of a 2-h perfusion was determined as described above.

The 24 testes perfused with medium containing fraction V secreted 17 ± 3.0 (s.e.m.) μg of testosterone per h. The results in Table 1 show that testosterone secretion by testes perfused with medium containing 4× crystalline BSA was similar to that obtained by paired testes receiving medium containing fraction V BSA. In contrast, testosterone secretion by testes perfused with medium either devoid of macromolecules or containing Dextran or ovalbumin, was only a quarter of that from testes receiving medium containing fraction V (Table 1).

Table 1 Effect of macromolecular constituents of perfusion medium on testosterone secretion by rabbit testes minus epididymides perfused *in vitro*

Macromolecule	% of Control*
4× recrystallised BSA	90 ± 8.1†
None	26 ± 3.5
Dextran T70	25 ± 4.2
Ovalbumin	26 ± 9.0

Testes were perfused for 2 h.

*Testosterone secretion was determined by measuring the testosterone content of testicular venous effluent collected during the second hour of perfusion and is expressed as a percentage of paired testis control perfused with medium containing fraction V BSA. Each value represents results from six rabbits.

†Standard error of the mean.

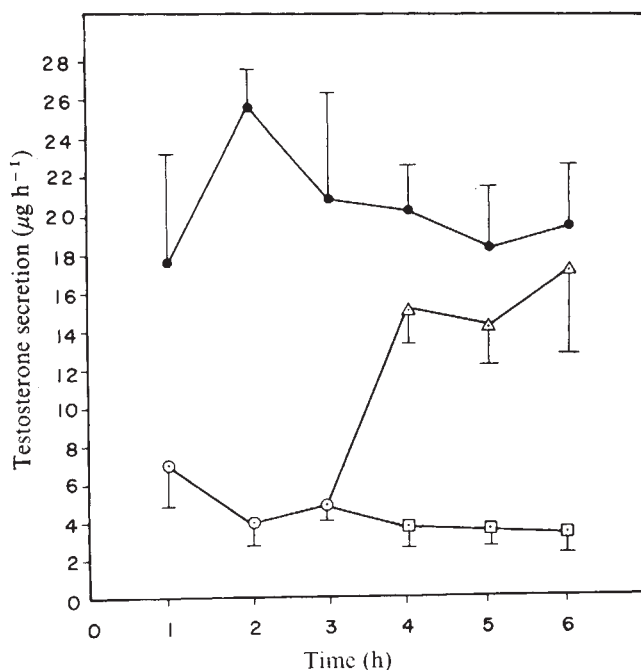


Fig. 1 Testosterone secretion by rabbit testes minus epididymides perfused *in vitro* with medium containing either 3% (w/v) fraction V BSA or 3% (w/v) Dextran. ●, Ten testes perfused with medium containing fraction V BSA for 6 h; ○, twenty testes perfused with the medium containing Dextran for the first, second and third hours of a 6-h perfusion experiment; △, ten testes switched to medium containing fraction V BSA during the fourth, fifth and sixth hours of perfusion. The latter ten testes were from the same rabbits that furnished the contralateral testes simultaneously perfused with medium containing fraction V BSA for 6 h. □, ten testes not switched but perfused with medium containing Dextran for 6 h. The $\pm$ above or below each point represents the standard error of the mean.

Substitution of Dextran or ovalbumin for BSA maintains the plasma osmotic pressure, preventing tissue swelling during perfusion. Thus it is unlikely that the diminution in testosterone secretion in the absence of BSA was due to some nonspecific effect on tissue viability. The high rate of secretion by testes perfused with medium containing fraction V BSA was not explained by the presence of a macromolecular contaminant in fraction V BSA since the 4 $\times$ crystalline BSA, which also supported a high rate of testosterone secretion, gave a single band on gel electrophoresis.

The unlikely possibility that the lipid present in BSA but absent from Dextran served as a substrate for steroidogenesis was eliminated by showing that testosterone secretion was similar in eight testes perfused with medium containing Dextran ($2.9 \pm 0.3 \mu\text{g}$ of testosterone per h), and in eight testes perfused with the same medium plus the residue of a chloroform-methanol extract of an equivalent amount of medium containing 3% fraction V BSA ($2.9 \pm 0.4 \mu\text{g}$ of testosterone per h).

Because these experiments were of short duration, the results could not eliminate the possibility that diminished testosterone secretion in the absence of albumin was only a transient phenomenon, or that reduced testosterone secretion in the absence of albumin resulted from some irreversible change in the steroidogenic cells. We therefore perfused one testis from each of 10 rabbits for 6 h with medium containing 3% fraction V BSA. The contralateral testis from each rabbit was perfused simultaneously with medium containing 3% Dextran for 3 h; then with medium containing 3% fraction V for a further 3 h. Finally, one testis from each of 10 more rabbits was perfused with medium containing Dextran for 6 h. Hourly samples of

venous effluent were collected and testosterone was quantified as described above. Figure 1 shows that testes perfused with medium containing fraction V BSA secreted $19 \pm 3 \mu\text{g}$ of testosterone per h in 6 h. In contrast, testes perfused with artificial medium containing Dextran secreted $4.5 \pm 0.7 \mu\text{g}$ of testosterone per h in 6 h. Importantly, the low testosterone secretion, obtained when testes were perfused with Dextran containing medium for 3 h, returned to the control rate of secretion after being changed to medium containing fraction V BSA (Figure 1). These results show that the low rate of secretion in the presence of Dextran did not increase spontaneously during 6 h of perfusion. Moreover, Dextran did not cause irreversible damage to the testicular steroidogenic cell within 3 h.

It can be calculated from the results in Fig. 1 that testes perfused with medium containing either BSA or Dextran produced $114 \mu\text{g}$ of testosterone ($19 \mu\text{g h}^{-1}$ for 6 h) or $27 \mu\text{g}$ of testosterone ($4.5 \mu\text{g h}^{-1}$ for 6 h), respectively, during 6 h of perfusion. The testosterone content of testes perfused with medium containing either BSA or Dextran for 6 h was $2.0 \pm 0.3 \mu\text{g}$ and $3.5 \pm 0.7 \mu\text{g}$ of testosterone per testis, respectively. This difference of $1.5 \mu\text{g}$ in testicular content fails to account for the difference of $87 \mu\text{g}$ in the amount of testosterone secreted by testes perfused with medium containing BSA, rather than with Dextran. Similarly, the testicular content of 5α androstan- 17β -ol-3-one, 5α androstan- $3\alpha,17\beta$ -diol, 5α androstan $3\beta,17\beta$ -diol and Δ^4 androst- $3,17$ -dione failed to account for significant amounts of the $87\text{-}\mu\text{g}$ discrepancy in testosterone secretion by testes perfused with medium containing Dextran rather than fraction V BSA. Therefore, diminished testosterone secretion in the absence of BSA cannot be explained by a failure of testosterone to be released from the testis or by an intratesticular accumulation of testosterone or some common testosterone metabolites.

These are the first experimental results to confirm Westphal's¹² hypothesis that, "binding proteins facilitate the passage of the (predominantly hydrophobic) steroids through the endothelium of the capillary walls". Further experimentation is required: to determine the relative impact of testosterone binding to erythrocytes, albumin or (sex-hormone-binding) globulin on testosterone secretion; to determine the mechanism by which testosterone binding to blood plasma macromolecules increases testosterone secretion; and finally to determine the effect of experimental treatments which alter either the concentration or steroid binding characteristics of blood plasma macromolecules on testosterone secretion.

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Recognition of lysosomal glycosidases *in vivo* inhibited by modified glycoproteins

PURIFIED liver lysosomal glycosidases display very short plasma survival times following intravenous injection. The mechanism by which these hydrolases are cleared from plasma involves specific recognition of the enzymes by way of sites associated with the enzyme molecules. In our study, a modified plasma glycoprotein terminating in *N*-acetyl-glucosamine has been shown to inhibit recognition and clearance of several purified lysosomal glycosidases.

Lysosomal glycosidases are glycoproteins^{1,2} mainly intracellular³ with only trace amounts normally occurring in plasma and extracellular fluids. Recent work⁴⁻⁷ indicates that various highly purified lysosomal glycosidases are very rapidly cleared from the circulation after intravenous administration. Rapid plasma clearance of lysosomal glycosidases seems to be mediated by liver plasma membrane binding sites or receptors⁸ with subsequent endocytosis and incorporation into liver lysosomes⁷. Rapid clearance is not a characteristic of all forms of a given glycosidase since some species (for example, purified normal serum β -glucuronidase)⁷ are not cleared and display relatively long plasma half lives. Moreover, the administration of diisopropylfluorophosphate⁶ rapidly raises the concentration of slow clearance forms of β -glucuronidase in rat plasma.

Extracellular survival of many glycoproteins is determined by the exposed or terminal sugar residues associated with the carbohydrate chains⁹. Nearly all plasma glycoproteins, for instance, having galactose as the penultimate sugar are rapidly cleared from plasma when the terminal sugar (sialic acid) is removed¹⁰. The mechanism of clearance of various asialo-glycoproteins with exposed galactose residues involves binding to a liver parenchymal cell plasma membrane receptor¹¹. More recently, a second clearance route, dependent on previous sequential removal of two sugars (sialic acid and galactose) from the carbohydrate chains of orosomucoid, has been described^{12,13}. Enzymatic removal of two sugars from orosomucoid results in the exposure of *N*-acetyl glucosamine. Plasma clearance of agalacto-orosomucoid seems to be mediated by recognition of terminal *N*-acetyl-glucosaminyl residues.

Several lines of evidence suggest that carbohydrate residues are important in mediating the clearance of lysosomal glycosidases¹⁴. In the hope of identifying the recognition site(s) associated with rapidly-cleared glycosidases, clearance competition experiments were undertaken using a family of purified rat liver lysosomal hydrolases including β -glucuronidase, β -galactosidase, *N*-acetyl- β -D-glucosaminidase and α -fucosidase. Asialo-orosomucoid (ASOR) and agalacto-orosomucoid (AGOR) were used as antagonists.

Clearance of various glycosidase activities was followed in the anaesthetised rat after a brief intravenous infusion of enzyme. The enzyme preparations were prepared from rat liver lysosomes and rat preputial gland. Rat preputial gland is an extremely rich source of β -glucuronidase from which large quantities of highly purified enzymes can be isolated. β -Glucuronidase from rat preputial gland and rat liver lysosomes are indistinguishable on the basis of catalytic properties⁷, molecular dimensions^{15,16} and clearance following intravenous infusion⁷.

Rat preputial gland β -glucuronidase, purified by the method of Ohtsuka and Wakabayashi¹⁷ had a specific activity of 2,000 phenolphthalein glucuronic acid (PGA) units mg^{-1} (see Table 1). Liver lysosomal β -glucuronidase, isolated by a modification of the method of Stahl and Touster¹⁸ using an affinity chromatography step described by Owens *et al.*¹⁸, had a specific activity of 1,000 PGA units mg^{-1} . β -Galactosidase was isolated from rat liver lysosomes with subsequent purification on concanavalin A-Sepharose as described by Stahl *et al.*⁷ and *p*-aminophenyl- β -D-thiogalactosyl-Sepharose as described by Distler and Jourdan¹⁹. The preparation used had a specific activity of 50 *p*-nitrophenyl (PNP) units mg^{-1} . α -Fucosidase, from rat liver lysosomes, was purified by affinity chromatography using ξ -amino-caproyl-fucosamine-Sepharose (Fucosylex, Miles). *N*-acetyl- β -D-glucosaminidase was isolated from rat liver lysosomes, as described by Stahl *et al.*⁷, to a specific activity of 880 PNP units mg^{-1} .

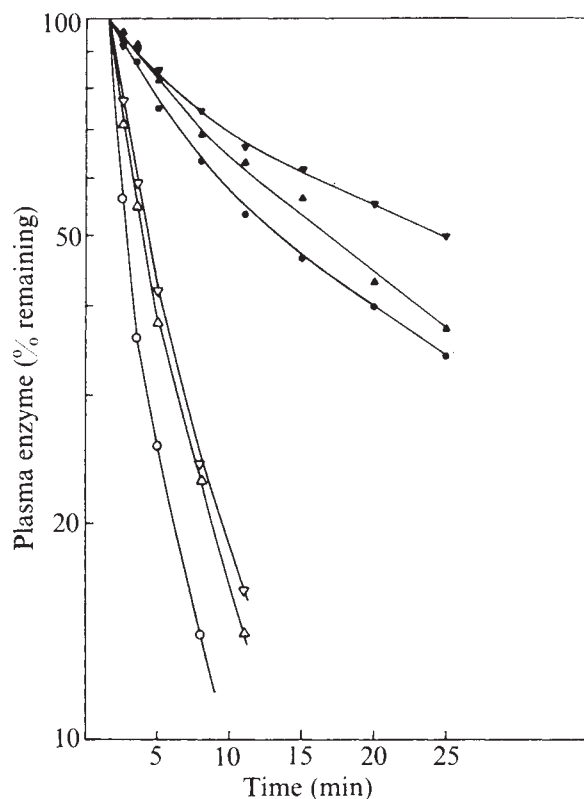


Fig. 1 Effect of AGOR on the clearance of liver lysosomal β -glucuronidase, β -galactosidase, and NAGA. Enzyme was infused as described in Table 1 with and without 1.0 mg AGOR. Plasma enzyme levels are expressed, after subtraction of pre-infusion plasma levels, as a percentage of the 1.5-min sample. ○, β -Glucuronidase; △, β -galactosidase; ▽, *N*-acetyl- β -glucosaminidase; ●, β -glucuronidase + AGOR; ▲, β -galactosidase + AGOR; ▼, *N*-acetyl- β -glucosaminidase + AGOR.

All enzymes were administered in 0.15 M NaCl. Asialo-orosomucoid was prepared by digestion of purified orosomucoid with *Clostridium perfringens* neuraminidase. The latter was protease-free. Agalacto-orosomucoid was prepared from asialo-orosomucoid by treating the latter with β -galactosidase isolated from the culture fluid of *Diplococcus pneumoniae*.

Clearance of three liver lysosomal glycosidases, β -glucuronidase, β -galactosidase and *N*-acetyl- β -D-glucosaminidase is summarised in Fig. 1. Two other enzymes, rat preputial β -glucuronidase and liver lysosomal α -fucosidase were also studied (Table 1). Clearance profiles in Fig. 1, constructed from analysis of sequential arterial plasma samples, demonstrate the very rapid nature of enzyme

Table 1 Effect of agalacto-orosomucoid and asialo-orosomucoid on the clearance of various lysosomal hydrolases

Enzyme	Inhibitor	Plasma level (units ml ⁻¹)				Plasma half life
		0 min	1.5 min	5.0 min	30 min	
L-β-Glucuronidase	—	0.11	10.6	2.8	0.14	1.3
L-β-Glucuronidase	AGOR	0.33	14.3	10.7	3.9	11.8
—	AGOR	0.16	0.2	0.2	0.2	
P-β-Glucuronidase	—	0.07	4.1	2.7	0.29	4.0
P-β-Glucuronidase	ASOR	0.14	5.6	3.0	0.85	4.0
P-β-Glucuronidase	AGOR	0.14	6.7	6.3	3.5	> 30
NAGA	—	0.49	2.5	1.1	0.04	2.8
NAGA	ASOR	0.36	2.6	0.9	0.09	3.0
NAGA	AGOR	0.54	5.0	4.2	2.0	23.0
—	AGOR	0.51	0.5	0.6	0.8	
β-Galactosidase	—	0.17	1.2	0.47	< 0.01	2.5
β-Galactosidase	ASOR	0.14	1.2	0.63	0.14	4.0
β-Galactosidase	AGOR	0.09	1.5	1.3	0.44	15.0
—	AGOR	0.2	0.2	0.1	0.1	
α-Fucosidase	—	1.3	2.0	1.7	0.5	14.5
α-Fucosidase	ASOR	0.9	2.0	1.5	0.5	12.5
α-Fucosidase	AGOR	1.2	2.5	2.4	1.5	36.0
—	AGOR	0.7	0.6	0.6	0.8	

Lysosomal (L) β-glucuronidase, 20 PGA units (20 μg), preputial gland (P) β-glucuronidase, 10 PGA units (5 μg), β-galactosidase, 5 PNP units (100 μg), *N*-acetyl-β-D-glucosaminidase, 20 PNP units (22 μg); and α-L-fucosidase, 5 PNP units (11 μg) were infused by femoral vein cannula into the anaesthetised rat without (—) or with ASOR (1 mg) or AGOR (1 mg), respectively, in a total volume of 0.5 ml. Female Wistar rats (50–60 g), anaesthetised with sodium pentobarbital (30 mg kg⁻¹, intraperitoneally), were used throughout. Femoral vein and carotid artery cannulations were made and enzyme preparations were infused for 70 s. The first blood sample was taken at 90 s using heparinised haematocrit tubes. Further details are described by Stahl *et al.*⁷. β-Glucuronidase was assayed using PGA¹⁵ or 4-methylumbelliferyl-(MU)-glucuronide as described by Stahl *et al.*⁷. β-Galactosidase was assayed using 2.5 mM PNP-β-D-galactoside or MU-β-D-galactoside. The reaction was run in 0.1 M Na acetate pH 4.3, containing 0.5 M NaCl and terminated by the addition of alkaline stopping reagent¹⁵. *N*-acetyl-β-D-glucosaminidase and α-fucosidase were assayed using the appropriate PNP- and MU-glycosides as described by Stahl *et al.*⁷. All units of enzymatic activity are expressed as μmol product formed per h. Protein was determined by the Miller method²⁰. The results are the average of two animals per enzyme determination. Results for AGOR (1 mg) infusion are from one animal only. Plasma enzyme levels are expressed as MU units per ml plasma. Preinfusion control plasma enzyme levels have been subtracted from post-infusion values. Plasma half life was estimated as the time required to reach 50% of the 1.5-min plasma level. NAGA = *N*-acetyl-β-D-glucosaminidase.

clearance. The actual plasma enzyme levels at selected post-infusion time points appear in Table 1.

When various lysosomal glycosidases were infused with 1 mg AGOR, the enzyme clearance curves were shifted considerably to the right (Fig. 1). As expected, plasma half life for each enzyme was appreciably extended and plasma enzyme levels 30 min after infusion were enhanced many times (Table 2). Injection of 1 mg AGOR alone (Table 2) had no effect on plasma glycosidase levels. Corresponding experiments were undertaken to determine whether ASOR has any inhibitory effects on enzyme clearance. ASOR (1 mg) had no effect on clearance of the five lysosomal enzyme preparations (Table 2).

To test whether inhibition of clearance of β-glucuronidase is dependent on the concentration of inhibitor, a series of doses of AGOR were administered with a tracer dose (0.005 mg) of preputial β-glucuronidase. The rate of enzyme clearance was measured as described previously⁷. Briefly, for each dose of AGOR, a semi-log plot was made of plasma enzyme level against time. The initial enzyme clearance rate was estimated from the slope of a line drawn through the first few (2–4) time points. Clearance rates,

expressed as units ml⁻¹ min⁻¹, are summarised in Table 2 for a series of doses of AGOR. As little as 30 μg of AGOR reduced β-glucuronidase clearance by 25%. The dose-dependent nature of AGOR inhibition of preputial β-glucuronidase clearance suggests that the two are competing for the same receptor site. However, this point will require more kinetic and structural analysis.

Previous work from our laboratory¹⁴ with two rat lysosomal glycosidases, β-glucuronidase and *N*-acetyl-β-D-glucosaminidase, indicated that periodate oxidation of the enzymes resulted in loss of rapid clearance without appreciable changes in catalytic activity. These results, along with those of Hickman *et al.*²¹, suggest that sugar residues are important in enzyme recognition and uptake by cells. The observations reported in the present paper where an *N*-acetyl-glucosaminyl terminal glycoprotein inhibited clearance of a family of lysosomal glycosidases support and extend earlier work implicating carbohydrate residues in the recognition site. Further, the results suggest that the recognition site includes *N*-acetyl glucosamine. Confirmation of this must await chemical analysis of glycopeptides derived from lysosomal enzymes. Of considerable interest are the recent findings of Achord, Brot, Gonzales-Noriega and Sly (in preparation), that highly purified human placental β-glucuronidase is rapidly cleared from the circulation in the rat and that the clearance system seems to recognise the carbohydrate moiety of the enzyme. Our results would suggest that the liver recognition system, described in mammals by Stockert *et al.*¹² and in birds by Lunney and Ashwell¹³ and Regoezi *et al.*²² for modified glycoproteins, may function to maintain the very low levels of lysosomal enzymes found in extracellular fluids. The results may prove to be important in enzyme replacement therapy for lysosomal enzyme deficiency storage diseases.

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Table 2 Effect of agalacto-orosomucoid on β-glucuronidase clearance rate

Enzyme (units)	Inhibitor (mg)	β-Glucuronidase clearance rate (units ml ⁻¹ min ⁻¹)
β-Glucuronidase (10)	—	0.55
	AGOR (0.03)	0.32
	AGOR (0.1)	0.30
	AGOR (1.0)	0.21

The effect of AGOR on the initial plasma clearance rate of preputial β-glucuronidase (10 PGA units) was estimated by plotting plasma enzyme levels after termination of infusion, as described in Table 1, against time. The rate was calculated from the slope of a line connecting the first few (2–4) points on the clearance curve as described in ref. 7. Various amounts of AGOR were added to the infusate.

human β -glucuronidase before publication. We also thank Dr Gilbert Ashwell, for providing ASOR and AGOR and for helpful suggestions, and Dr Oscar Touster, Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee (Opheim and Touster, in preparation), for providing the method of purifying α -fucosidase.

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Model for the structure of the gastric mucous gel

THE SURFACE of the stomach is covered by a layer of mucous gel which protects the underlying mucosa from the harmful, acidic stomach contents. The principal component of the gel has been isolated from pig gastric mucus, purified, and shown to be a glycoprotein of molecular weight 2×10^6 (Table 1)^{1,2}. This glycoprotein consists of four equal sized subunits (molecular weight 5×10^5) joined by disulphide bridges^{3,4}. Each glycoprotein subunit consists of a protein core, 14% by weight of the glycoprotein, with carbohydrate side chains attached. The protein core consists of two regions, one rich in serine, threonine and proline and bearing all the carbohydrate, the other having an amino acid composition characteristic of a globular protein. The latter contains cystine residues which bridge the four subunits. Mild reducing agents and proteolytic enzymes each split the glycoprotein into four subunits^{4,5}. The carbohydrate chains, approximately 15 residues in a branched structure, account for 82% by weight of the glycoprotein and carry ester sulphate residues. The problem therefore is to explain how such a glycoprotein molecule can associate to form a gel which *in vivo* is essentially impermeable to proteolytic enzymes and which can act as a barrier to protons probably by supporting a pH gradient.

The mucous gel isolated directly from the stomach is clearly viscoelastic, despite its non-uniform concentration. It can be completely solubilised by guanidinium chloride or by homogenisation in water, demonstrating that the gel is stabilised solely by non-covalent interactions between the component glycoprotein molecules⁶. The solubilised gastric glycoprotein, in 0.2 M KCl, has a high intrinsic viscosity of 320 ml g^{-1} but

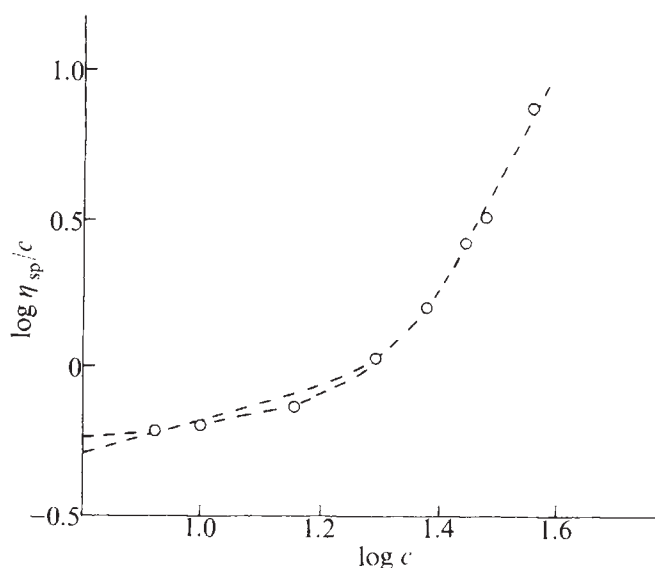


Fig. 1 The dependence of the viscosity of the gastric glycoprotein on concentration: plot of $\log \eta_{sp}/c$ against $\log c$. Viscosities were measured using a Couette viscometer. Solvent: 0.2 M KCl, 0.02% (w/v) azide; 0.02 M K acetate buffer, pH 5.5.

the concentration dependence of its viscous, sedimentation and diffusion properties indicate a surprisingly low degree of intermolecular interaction (see Table 1) (Fig. 1). At higher glycoprotein concentrations of $20\text{--}30 \text{ mg ml}^{-1}$ the viscosity indicates a deviation from this behaviour and intermolecular interaction assumes an increasingly significant contribution to the viscosity of the solution. This is demonstrated by the logarithmic plot of reduced specific viscosity against concentration (Fig. 1), where the increase in slope on going from low to higher concentrations of glycoprotein is characteristic of the behaviour of certain interacting synthetic polymers⁷. The major change in behaviour occurs in the concentration range $20\text{--}25 \text{ mg ml}^{-1}$. Concentrating the glycoprotein further will result in gel formation *in vitro*, although the nature of this gel and the exact conditions for its formation have yet to be studied in detail. The average concentration of the glycoprotein found in mucous gel isolated directly from the stomach is in the region of $30\text{--}40 \text{ mg ml}^{-1}$, that is, in the range in which intermolecular interaction is shown above to be occurring.

The flow properties of the glycoprotein at low concentrations in 0.2 M KCl solution can be accounted for by a molecule which is highly expanded and approximately spherical⁸. An indication of the solution volume occupied by the glycoprotein at high dilution is given by its effective hydrodynamic volume, V_e , from the relationship

$$V_e = (f/6\pi\eta_0)^3(4\pi\mathcal{N}/3M)$$

assuming the molecule is spherical. f is the frictional coefficient calculated from the diffusion coefficient, η_0 is the viscosity of the solvent, $\mathcal{N}$ is Avogadro's number and M the molecular weight. For the glycoprotein in 0.2 M KCl, V_e has a value of 40 ml g^{-1} . V_e does not define the precise geometry of the molecule itself⁸ but it does give an indication of the volume within which the probability of intermolecular interaction becomes appreciable. On this basis, therefore, the sphere of interaction of glycoprotein molecules will spread over the total solution volume at a solute concentration of 25 mg ml^{-1} . This, it will be noted, is in the concentration range at which independent experimental data shows intermolecular interaction to become significant (Fig. 1). A large volume of solvent is thus enclosed by the glycoprotein molecule and it is quite possible therefore to account for the existence of solutions of concentration higher than 25 mg ml^{-1} by an overlap of molecular volumes or interdigitation. This would, however, reduce the

Table 1 Properties of the glycoprotein from pig gastric mucus

Chemical composition ⁴	(% by weight of freeze-dried glycoprotein)	
Carbohydrate 73.6	galactose (24.9), glucosamine (23.3), galactosamine (8.7), fucose (14.3), <i>N</i> -acetylneuraminic acid (2.4).	
Protein 12.9	Sulphate 3.1	Water 10.4 (by difference)
Physical data ²	(in 0.2 M KCl; 0.02 M K acetate; 0.02% (w/v) Na azide; pH 5.5).	
$s_{25,w}^0$	18.7S	
$D_{25,w}^0$	$0.69 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$	
Molecular weight	2×10^6	
f	$5.96 \times 10^{-7} \text{ cm s}^{-1}$	
V_e	40 ml g ⁻¹	
Concentration dependence of physical parameters:		
Sedimentation coefficient	$K_s [= s^0 d(s^{-1})/dc]$	260 ml g ⁻¹
Diffusion coefficient	$K_D [= -(1/D^0) dD/dc]$	0 ml g ⁻¹
Viscosity (Huggin's constant)	$k [= (\eta_{sp}/c - [\eta])(1/[\eta]^2)c]$	0.3

Sugars were analysed by gas-liquid chromatography and the protein is a summation of the contents of the individual amino acids.

rotational relaxation of the molecule, lead to a rapid rise in viscosity and, at some point depending on the nature and strength of non-covalent intermolecular forces, to gel formation. This point is further supported by calculations of the maximum volume which could be occupied by the glycosylated part (< 90% by weight) of the molecule assuming fully extended sugar chains eight residues in length⁹ forming a cylinder on four extended glycosylated polypeptide chains each 565 residues long. This model leads to a volume of 12 ml g⁻¹ which is a substantial proportion of the effective hydrodynamic volume V_e . The shortfall between this value and that of 40 ml g⁻¹ found experimentally suggests that the four glycoprotein subunits may be flexible about the non-glycosylated disulphide bonded regions of the polypeptide chains.

Based on the above evidence we propose the following model for the structure of the gastric mucous gel. The gel is composed of highly expanded, solution-filling, glycoprotein molecules which, packed together, interact at or near their surfaces. A large proportion of the solvent is thus intra-molecular and the polymer extends more or less evenly throughout the gel. This model contrasts with those structures elucidated for other polysaccharide gels such as agar¹⁰ where a much lower concentration of solute can form a relatively low density open network of molecular fibres. The gastric mucous gel, therefore, would be expected to be relatively impermeable to large molecules such as enzymes compared with the free diffusion of such through agar gels. Further, such a structure should reduce considerably any turbulence in the solvent within the gel allowing a relatively stable gradient of pH to be maintained at the surface of the stomach.

This model predicts that gel formation at a given concentration should be markedly dependent on the effective volume in solution of the glycoprotein molecule. Factors such as ionic strength which have been shown to change the size of V_e do in fact markedly affect the intermolecular interactions². It is not yet clear what could stimulate intermolecular interaction between polar chemical groups which appear to be strongly solvated, although there is evidence that removal of water from such structures brings about preferential interaction between sugar chains¹⁰.

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Cloning of all *EcoRI* fragments from phage λ in *E. coli*

CLONING of DNA fragments is a new powerful technique in molecular genetics and a number of interesting experiments involving both prokaryotic and eukaryotic genomes have already been reported (see ref. 1 for review). We present here results concerning the cloning of all *EcoRI* (ref. 2) fragments from phage λ DNA in *Escherichia coli* using as a vector plasmid pSC101 (ref. 3). To achieve this, we had to solve the problem of propagating the λ fragment containing the immunity region since the phage strain used had a temperature-sensitive mutation in that region.

DNA from the lysogenising strain c1857 of phage λ and the supercoiled form of plasmid pSC101, were digested at 37 °C with restriction enzyme *EcoRI* (prepared according to a modification of the method of Yoshimori⁴) in 100 mM Tris, pH 7.5, and 10 mM MgCl₂ (ref. 5). After digestion was complete, as judged by electrophoresis of the DNAs, *EcoRI* was inactivated by heating the incubation mixtures at 65 °C for 5 min. The two digests were then mixed together, slowly chilled to 0 °C to allow reannealing of the "sticky ends" formed by *EcoRI* to occur and incubated for 16 h at 0 °C and 1 h at 14 °C in 50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 0.035 mM ATP, 10 mM dithiothreitol⁶ and polynucleotide ligase from T4-infected *E. coli* (from Miles or from Dr F. Rougeon). The ligated mixture was used to transform strain HB 101 (ref. 7) of *E. coli*. The transfectants were plated on L agar⁸ containing 15 (or 10, ref. 9) $\mu\text{g ml}^{-1}$ tetracycline, grown overnight at 37 °C and further purified on plates containing tetracycline. Selection of recombinant clones was carried out either by a group screening using gel electrophoresis, or by the hybridisation technique of Grunstein and Hogness¹⁰.

In an experiment, in which 85 clones were screened in groups of 7-8, at least 10 were found to contain λ fragments 3, 4 or 5 (the numbering of the six *EcoRI* λ fragments is according to decreasing molecular weight¹¹);

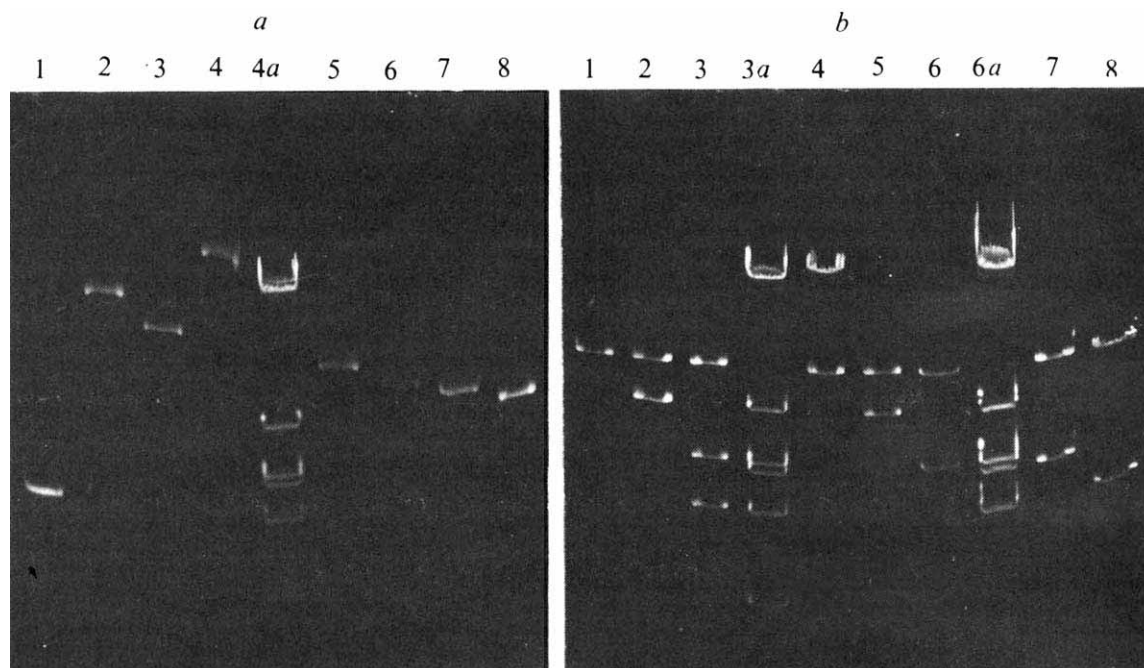


Fig. 1. Electrophoresis of recombinant pSC101- λ fragment plasmids before (a) and after (b) *EcoRI* digestion. a, Slots contain: 1, supercoiled circular pSC101; 2, pSC101 + fragment 2 (two copies); 3, pSC101 + fragments 3+5; 4, pSC101 + fragments 1+6; 4a, *EcoRI* fragments of λ DNA; 5, pSC101 + fragment 2; 6, pSC101 + fragment 3; 7, pSC101 + fragment 4; 8, pSC101 + fragment 5. b, Slots 1-8 contain the same plasmids as in a after digestion with *EcoRI*; slots 3a and 6a contain *EcoRI* digests of λ DNA; in slot 3a fragments were heated for 5 min at 70 °C to show the separation of fragments 1 and 6, which gives a single band in non-heated *EcoRI* fragments (slot 6a).

these range in molecular weight from 3.5 to 3×10^6 . One additional clone contained two λ fragments, 3 and 5, which are non-adjacent in the genome and account together for 6.5×10^6 of DNA. Still another clone contained fragments 1 and 6; these fragments are bound together through the natural cohesive ends of λ DNA; they contain the whole left arm of the genome and have a molecular weight of 16×10^6 ; obviously, these fragments cannot be inserted separately since each one of them lacks an *EcoRI* sticky end in the linear form of λ DNA. The easier integration of low molecular weight compared to high molecular weight fragments is expected; our failure to find recombinants harbouring λ fragment 2, which has a molecular weight of 4×10^6 , was, therefore, at first sight, surprising.

An experiment in which λ fragment 2 was eluted from Agarose¹², ligated with pSC101 and used for transfection, did not yield any recombinant plasmid, in spite of the fact that 600 colonies were examined by the hybridisation technique; in contrast, a parallel experiment with fragments 3 and 4 was successful; this suggests that our negative results with fragment 2 were not due to the fact that fragments eluted from Agarose cannot be ligated.

Since fragment 2 comprises the λ immunity region, which is the locus of the temperature-sensitive mutation that induces the lysogen at 37 °C, an experiment was carried out in which Agarose-eluted fragment 2 was ligated to pSC101 and transfectants were grown overnight at the permissive temperature of 30 °C. In this case, 4 out of 600 transfectants were found to be positive by the hybridisation test. The plasmids prepared from three colonies contained a single copy of fragment 2, whereas the fourth clone contained two copies of this fragment.

It may be interesting to remark that recombinant plasmids containing λ fragments may themselves be useful in that they represent new vectors carrying restriction sites brought in by the λ fragments which may not exist in the parent plasmids.

Figure 1 presents the gel electrophoresis results obtained with the recombinant plasmids described above, before and after *EcoRI* digestion.

These experiments were carried out in low-risk containment conditions, as defined by the Asilomar guidelines. All the clones described in the present paper are available on request.

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Azide-dependent mutants in *E. coli* K12

SODIUM azide is known specifically to inhibit or activate a number of enzymatic activities from bacteria¹ and from higher organisms^{2,3}. Furthermore it has been shown that azide can inhibit the Mg^{2+} -dependent ATPase of *Escherichia*

Table 1 Classification of *E. coli* mutants

	I (ADA)	II (ADB)	III (ADC)	IV (ADD)
Number of mutants	1	1	3	3
Azide replaced by	Nothing	Nothing	Threonine or pyridoxine	Phenylalanine, tyrosine and tryptophan*
Location of the mutation (Taylor's map)	Around 7 min†	Around 20 min†	0 min- <i>thrC</i>	Around 5 min†

The parental strain is CSH 57A (ref. 6). Ultraviolet mutagenesis (10^{-2} – 10^{-3} survival) was used.

*The parental strain is Trp⁻. The conditional requirement for all three aromatic amino acids was tested on an ADD mutant which received the wild-type *trp* operon by transduction.

†Approximate mapping determined by interrupted conjugation.

*coli in vivo*⁴, at concentrations that do not produce any deleterious uncoupling effects on the membrane energy-transducing apparatus of the cell⁵. We felt that those facts could provide the basis for searching out a new type of mutation in *E. coli*—azide dependency.

The selection procedure involved penicillin treatment in a liquid minimal medium devoid of azide, followed by a second enrichment step given by the presumed slower growth of the mutants on a solid minimal medium containing 2×10^{-3} M azide. Eight independent mutants were isolated and separated into four classes according to the specific replacement of azide for growth and the location of the mutation on the *E. coli* chromosome (Table 1).

Two of the mutants (ADA 1 and ADB 1) have an absolute requirement for azide. Although they exhibit an identical phenotype, conjugation experiments show that they have distinct locations, mapping at about 7 and 20 min respectively on the *E. coli* chromosome. The ADA 1 mutation was shown to be recessive to the wild-type allele. If exponential cultures from either mutant strain were transferred in growth medium devoid of azide all growth ceased in about two hours. Measurements of the kinetics of incorporation of tritiated uracil and ¹⁴C-L-tryptophan in TCA

insoluble material *in vivo* suggest a translational defect in both mutants.

The third class of mutants (ADC 1–3) includes Thr⁻ conditional mutants whose specific requirement for threonine can be replaced either by sodium azide or pyridoxine (Table 1). Mutations were cotransducible at high frequency with *pyrA* and *serB*, and thus presumably mapped in the threonine operon. This was confirmed by the use of a λ phage carrying the entire threonine operon⁷ and three derived λ phages each carrying a mutation in one of the three genes (*thrA*, or *thrB* or *thrC*)⁸: the ADC mutations tested were recessive and mapped precisely in the C gene of the operon. Threonine synthase—the C gene product—was then measured in crude extracts from one mutant strain grown on minimal medium containing azide (Table 2). Only 5% of the wild-type specific activity was found in this mutant. The mutant enzymatic activity *in vitro* was not stimulated by sodium azide; on the contrary azide gave a 15–20% inhibition, comparable to the inhibition obtained on the wild-type activity. Kinetics of irreversible thermal denaturation of the enzyme in crude extracts showed monophasic curves for wild-type and mutant activities but with different half lives: about 4.5 min for the wild type and 0.5 min for the mutant at 56 °C. This thermolability of the mutant enzyme compared with its wild-type counterpart seems to be a real property of the modified enzyme since a mixture of mutant and wild-type extracts showed biphasic kinetics compatible with the mere addition of the two separate kinetics. Interestingly enough, in crude extracts, azide potentialised the irreversible thermal denaturation of the wild-type enzyme, but had no such effect—nor any stabilising effect—on the mutant enzyme (Table 2). Once purified, however, the wild-type enzyme (prepared by B. Burr) showed a complex biphasic curve of denaturation kinetics with no measurable effect of azide. Threonine synthase from the parental and the mutant strains was partially purified and the affinity for pyridoxal-5'-phosphate measured. Estimation of dissociation constants for the coenzyme from Hill plots at $v = Vm/2$ gives a value of 1.2 μ M for the wild-type and 8.5 μ M for the mutant enzyme. Consistently, in the absence of any added pyridoxal-5'-phosphate, about 15% of the wild-type enzyme was found to be in the holoenzyme form whereas no such form could be detected in the mutant. Sodium azide 2×10^{-3} M or 2×10^{-2} M had no significant effect on the dissociation constant of the mutant enzyme for pyridoxal-5'-phosphate.

The last class behaves as pleiotropic conditional mutants. Their growth is dependent either on sodium azide, or on tyrosine, phenylalanine and tryptophan (Table 1). However, shikimic acid does not allow growth. One mutant (ADD 1) was mapped by interrupted conjugation: the gene involved is approximately at 5 min on Taylor's map. Reversion of the Tyr⁻ or Phe⁻ phenotype in all cases tested (more than 100) gave a pleiotropic reversion with the additional property of large excretion of both tyrosine and phenylalanine (reversion rate of about 10^{-5}). Enzymatic activities of the aromatic pathways were measured in crude extracts of two revertant strains grown on minimal medium in the absence of phenylalanine and tyrosine (in the presence of

Table 2 Thermosensitivity of threonine synthase in ADC 1 crude extract

			Wild type	Mutant ADC 1
Half life of thermal denaturation (min)	No addition	56 °C	4.5	0.5
		49.5 °C	8.5	5.5
	with NaN ₃	56 °C	3	—*
	2×10^{-3} M	49.5 °C	—*	6
	with NaN ₃	56 °C	2	—*
	2×10^{-2} M with NaCl	49.5 °C	—*	5.5
	2×10^{-2} M	56 °C	4.5	—

*Not done.

Bacteria were grown exponentially in minimal medium containing the growth factors of the strains (including isoleucine), glucose 0.4% and sodium azide 2×10^{-3} M. Crude extracts were prepared by sonication of the bacteria previously washed with a potassium phosphate buffer 2×10^{-2} M (pH 7.2). For threonine synthase assay, final concentrations in 0.1 ml of the reaction mixture were: 50 mM N-2-hydroxyethylpiperazine-N-2-ethane sulphate adjusted to pH 8 with KOH; 100 mM ammonium sulphate; 0.1 mM pyridoxal-5'-phosphate; 0.24 mg ml⁻¹ bovine serum albumin; and 1.3 mM (0.035 mCi mmol⁻¹) ¹⁴C-L-homoserine phosphate (prepared according to ref. 9). The reaction was carried out at 37 °C for 5–60 min. At the end of the reaction, 5 μ l of KH₂PO₄ 1 M and 0.9 ml of barium bromide (11 mg per ml 90% ethanol) were added, and the precipitated mixture was allowed to stand for 5 min at 4 °C. An aliquot of the supernatant was counted for radioactivity. The radioactivity found in the supernatant at 0 min reaction time was subtracted from the radioactivity measured (this background represents less than 2.5% of the total added radioactivity). At least 90% of the non-precipitating radioactive product was shown to be threonine by chromatography. The specific activity of the wild-type enzyme was 11.7 nmol min⁻¹ mg⁻¹ of proteins.

For thermal denaturation experiments, NaN₃ (or NaCl) was preincubated with the crude extract for 10 min at room temperature.

Table 3 Activities of the biosynthetic pathways of tyrosine and phenylalanine in ADD 1 mutant and two revertant strains

Growth medium	Wild type *	†	ADD 1 †	Revertant I from ADD 1 *	Revertant II from ADD 1 *
Prephenate dehydratase	7.5	8.0	12.5	19.0	11.0
Prephenate dehydrogenase	23.5	23.5	103.5	288.5	56.5
Chorismate mutases	34.0	33.5	100.0	335.5	75.5
DAHP synthetases	159.0	195.0	267.5	512.5	562.5

Crude extract preparation was as described in legend of Table 2. Buffer was potassium phosphate 2×10^{-2} M (pH 7.2). Prephenate dehydratase and chorismate mutase assays were done according to ref. 14. Prephenate dehydrogenase assay according to ref. 15. Units are $\mu\text{mol per min per mg proteins}$ ($\times 10^3$).

*Bacteria were grown exponentially on 63 minimal medium containing the growth factors of the strains and glucose 0.4%.

†Bacteria were grown exponentially on the above medium containing in addition sodium azide 2×10^{-3} M.

tryptophan) (Table 3). Relative to their wild-type counterparts, prephenate dehydratase activity, an enzyme specific to the phenylalanine pathway, was found to be 2.5 higher in strain I and 1.5 higher in strain II; prephenate dehydrogenase, an enzyme specific to the tyrosine pathway, showed an increase of about 12-fold in strain I and 2.5-fold in strain II; chorismate mutases, specific both for phenylalanine and tyrosine, increased about 10 times in strain I and 2.2 times in strain II. As to DAHP synthetases, the increase in both strains was more than threefold, being due largely (but not exclusively) to the tyrosine-sensitive DAHP synthetase increase. It should be noticed that the above enzymes concern three different operons¹⁰⁻¹³. ADD 1 mutant, grown in the presence of NaN_3 , similarly showed a significant increase in the above activities compared to the wild type grown in the same conditions (Table 3).

In summary, mutations in at least four different genes can result in azide dependency for growth. None map in the described *azi* gene which is responsible for azide sensitivity^{17,18}. Two genes, at distinct locations, appear to be concerned with the translational mechanism of the cell. No known genes of this type in *E. coli* have the required locations. The third class of mutants map in the *thrC* gene, and the fourth class gives rise to a conditional pleiotropic requirement for tyrosine, phenylalanine and tryptophan which has not been previously described.

Although the precise step or mode of action of sodium azide is still to be found, the search for azide-dependent mutants in *E. coli* seems to be rewarding, having given rise to the discovery of three possible new genes and one new allele of a known gene, and perhaps permitting the detection and study of other new genes and new gene products.

We wish to thank Georges N. Cohen for encouragement and stimulating discussions, A. Képès, P. Truffa-Bachi and I. Saint-Girons. The author is a recipient of a fellowship from the Délégation Générale à la Recherche Scientifique et Technique.

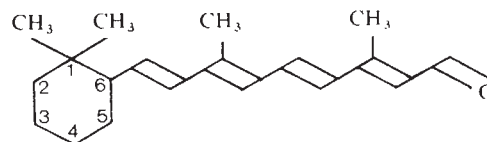
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Is proton transfer the initial photochemical process in vision?

THERE has been renewed effort¹ to test the so-called proton transfer hypothesis (PTH)² or hydrogen shift¹ formulation for the initial photochemical step in vision. This hypothesis proposes an alternative to photochemical *cis* to *trans* isomerisation of rhodopsin, while attempting to account for some of the spectral properties of bathorhodopsin (previously called prelumirhodopsin), particularly certain low frequency lines at 920, 877 and 856 cm^{-1} . Spectral lines in this region are not unique to the resonance Raman spectrum of bathorhodopsin³, however, for similar spectral absorption has been found with 11-*cis* retinal crystals⁴. The discovery that the batho-intermediate formed by irradiating chicken iodopsin at -195°C spontaneously reverted to iodopsin at -140°C by a dark, thermal process⁵, rather than decaying to lumirhodopsin, also seemed to require such an alternative hypothesis² (Fig. 1). Although an attempt to demonstrate proton transfer by means of tritium incorporation into the retinylidene chromophore failed², Fransen *et al.* apparently now confirm the PTH by their report¹ of a small, though significant incorporation of deuterium into retinal. Their newer experiments are similar in design to the earlier ones, differing mainly in their use of deuterium rather than tritium and their method of analysis of labelled retinal. The experiments described here bear directly on the proposed PTH and were performed with an analogue of retinal, 5-desmethyl (dm) retinal:



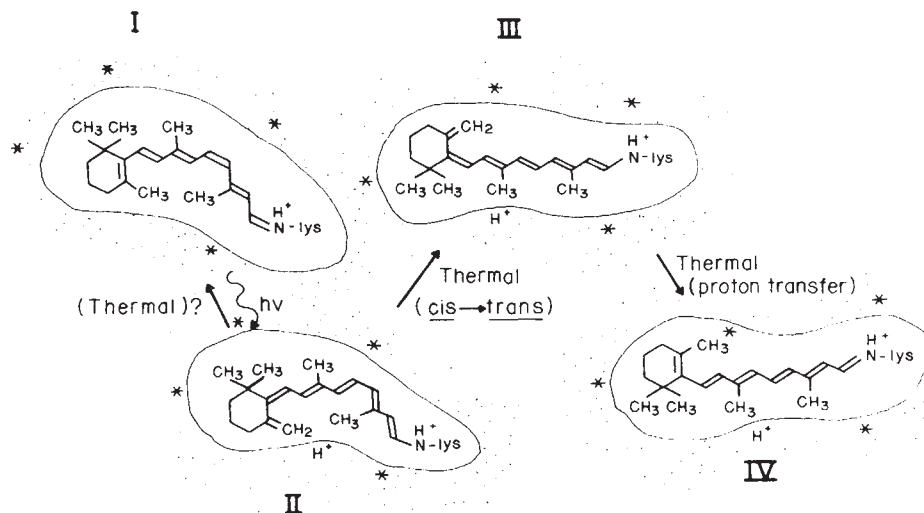
They show that the initial compound produced by irradiating a visual pigment, the batho-intermediate, cannot result from the transfer of a proton from the chromophore to opsin.

5-dm retinal was prepared during a study of the spectral properties of visual pigment analogues⁶. The presence of a chromophore bearing a hydrogen atom, rather than a methyl (CH_3) group, on the 5 position of the ionone ring

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Fig. 1 Hypothetical mechanism for photochemical proton transfer followed by thermal *cis* to *trans* isomerisation in a visual pigment. The native or regenerated visual pigment, is represented by structure (I). In the experiments referred to^{1,2}, * represents either deuterium or tritium atoms which have been introduced into opsin by incubation in either deuterated or tritiated water, respectively. After photochemical transformation to the batho-intermediate (II), incorporation of deuterium or tritium into the retinylidene chromophore can occur during either the thermal steps leading to the intermediate (IV), or in the hypothetical thermal return of the batho-pigment to (I). Presumably intermediate III is a short-lived transient.



should preclude the formation of the proposed batho-intermediate¹. In particular, the structure designated as (II) in Fig. 1 is impossible in a pigment containing the 5-dm chromophore.

5-dm retinal was synthesised as described earlier⁶ and then purified by high performance liquid chromatography (HPLC). Individual geometric isomers were isolated by HPLC following the procedure and with separations similar to those reported for the isomers of retinal⁷.

Identification of the 5-dm retinal isomers was based on their photochemical behaviour as well as their order of elution from silica gel, which seemed to be the same as that of *cis* and *trans* retinals. The spectral maxima of the individual 5-dm isomers are given in Table 1.

Table 1 Spectral maxima of all-*trans* and mono-*cis* isomers of 5-dm retinal

	(nm)	(nm)
all- <i>trans</i>	373	268 (0.13)
13- <i>cis</i>	368	268 (0.29)
11- <i>cis</i>	367	268 (0.67)
9- <i>cis</i>	367	273 (0.34)

The values given in parentheses are relative heights of the secondary peaks. For each isomer, the main absorption band is arbitrarily assigned an absorbance of 1.0 at the wavelength of maximum absorption. The solvent was hexane.

Each of the isomers was mixed with a digitonin solution of opsin which had been prepared from frozen cattle retinae⁸. Only the 11-*cis* and 9-*cis* isomers combined, in each case forming a pigment with $\lambda_{\max}$ at 480 nm but at rates approximately 100 times slower than observed with the corresponding isomers of retinal. Complete spectra and other properties of these artificial pigments, called 5-dm rhodopsin (11-*cis*) and 5-dm isorhodopsin (9-*cis*) will be presented elsewhere.

To determine the presence and properties of any intermediates during the photolysis of the 5-dm pigments, I studied the photochemical behaviour of 5-dm isorhodopsin at low temperatures, utilising the absorption vessel designed and built by Yoshizawa and Horiuchi⁹ for work at liquid nitrogen temperatures and above. 5-dm isorhodopsin which had been formed by reacting 9-*cis* 5-dm retinal with a digitonin solution of opsin was mixed thoroughly with twice its volume of glycerol. Measured in a 0.3-cm light path cell at 20 °C, the absorbance of the resulting solution was 0.5 at 480 nm. Figure 2a, curve 1 is the spectrum of 5-dm isorhodopsin at 191 °C. Some unreacted 9-*cis* 5-dm retinal remains as the oxime. Spectra resulting from violet light irradiation of the pigment in glycerol-aqueous glass are

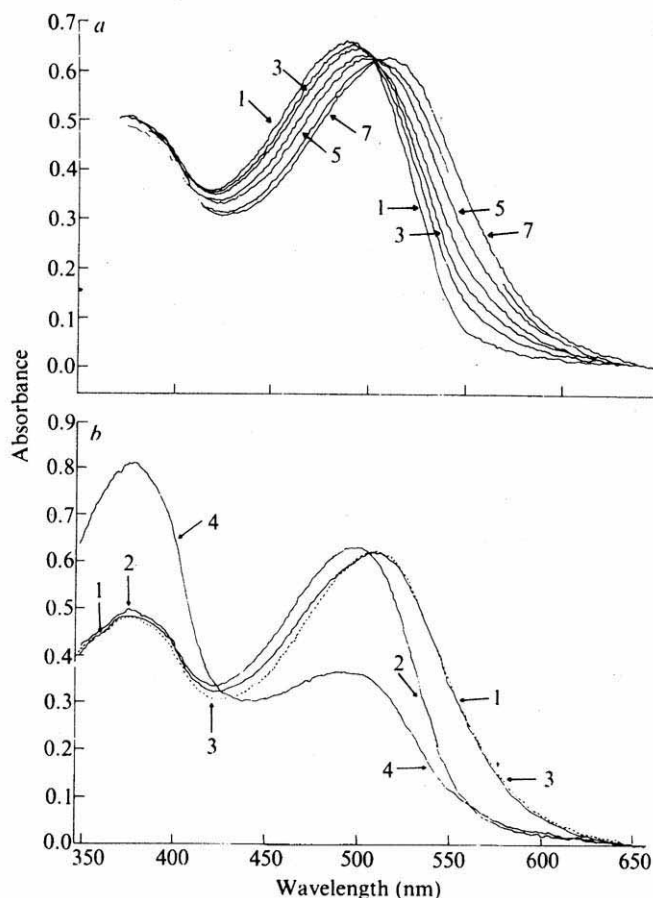


Fig. 2 a, The photochemical transformation of 5-dm isorhodopsin to 5-dm bathorhodopsin. 0.3 ml of a 67% glycerol solution of regenerated 5-dm isorhodopsin in approximately 1% digitonin and 0.01 M NH_2OH , pH 6.5, was placed in a cell with a 3-mm light path. The sample was cooled to -191°C and its spectrum recorded (1). The sample was then illuminated with violet light formed from the radiation of a high intensity xenon lamp which was passed through a 437-nm interference filter. Spectra 2-7 were recorded after total irradiation times of 2 s, 40 s, 80 s, 160 s, 320 s and 1,280 s, respectively. b, The reversible photochemical transformation among 5-dm batho-, 5 dm iso- and 5-dm rhodopsin. Conditions are the same as for a. 1, Same as spectrum (7) in a; 2, after irradiation with red light (600-nm cutoff filter) for 6.5 min; 3, after irradiation of (2) with violet light (437-nm interference filter) for 8 min; 4, after warming solution to $+20^\circ\text{C}$ and then recooling to -191°C .

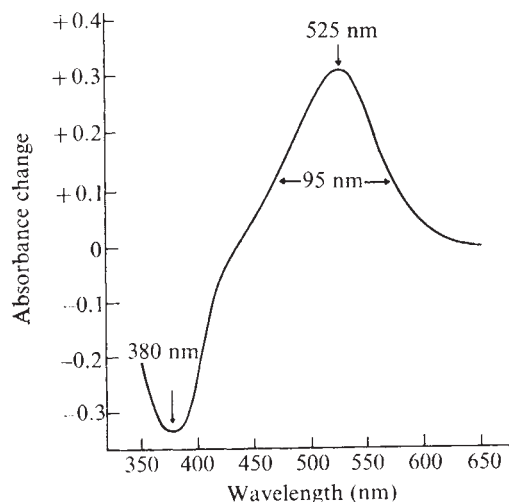


Fig. 3 Difference spectrum of 5-dm bathorhodopsin at -191°C . Conditions are given in Fig. 2a and b. The curve drawn results from subtracting spectral curve (4), from curve (3) in Fig. 2b.

shown in Fig. 2a, curves 2–7. The appearance of an isosbestic point at 503 nm argues that Fig. 2a illustrates the single-step transformation of 5-dm isorhodopsin into one, or a mixture of, long-wavelength absorbing intermediate(s).

Photoreversibility of this pigment system is shown in Fig. 2b, first by the transformation of the long wavelength absorbing intermediate(s) into shorter-wavelength-absorbing species, presumably 5-dm rhodopsin and 5-dm isorhodopsin. The first process (curves 1 and 2) is followed by the reformation of the long wavelength absorbing intermediate(s). Thus spectrum 3 of Fig. 2b contains thermostable pigments, presumably 5-dm rhodopsin and 5-dm isorhodopsin, as well as a thermally unstable component which decomposes to 5-dm retinal oxime and opsin when the solution is warmed to 20°C , as shown in spectrum 4. The difference spectrum of the thermally unstable compound, drawn in Fig. 3, has a positive peak with λ_{max} at 525 nm and a band width at half maximum of 95 nm. This spectrum characterises an intermediate which, because of its spectral and thermal analogies to cattle bathorhodopsin⁹, I call 5-dm bathorhodopsin.

When the violet irradiated mixture of 5-dm pigments (Fig. 2b, curve 3), containing 5-dm bathorhodopsin, and presumably 5-dm rhodopsin and 5-dm isorhodopsin, is warmed to only about -165°C , a decrease in absorbance at wavelengths greater than 500 nm and an increase in absorbance in the 400–500 nm region occurs. This change is consistent with the transformation of 5-dm bathorhodopsin into a pigment with λ_{max} at 497 nm and band width at half maximum of 80 nm. I call this pigment 5-dm lumirhodopsin. The spectrum of 5-dm lumirhodopsin and its formation from a longer wavelength precursor at around -165°C suggest it is analogous to cattle lumirhodopsin¹⁰.

These results show that a batho-intermediate can be formed from a pigment lacking an allylic methyl group in the ionone ring of the chromophore. Without such a methyl group it is impossible to transfer a hydrogen atom or ion from a group on the ionone ring to opsin and thereby form the structure proposed^{1,2} for bathorhodopsin. Since 9-dm pigments and 13-dm pigments also form batho-pigments at -190°C (my unpublished results), it seems reasonable to conclude that photochemical proton transfer from an allylic methyl-group on the chromophore to a site on opsin is not responsible for the rhodopsin (or isorhodopsin) to bathorhodopsin transition. *Cis* to *trans* isomerisation

still seems to be the process which can best account¹¹ for the experimental observations bearing on the initial photochemical event in the photochemistry of visual pigments, both natural and artificial.

This work was supported by a grant from the National Eye Institute. The low temperature experimentation was performed while I was a visiting professor in the Department of Biophysics of Kyoto University and I thank Professor T. Yoshizawa and his colleagues for hospitality and assistance. I also thank Professor B. Honig, Hebrew University of Jerusalem, for encouragement and R. B. Kropf for helpful emendations in the manuscript.

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Corrigendum

In the article “Perception of melodies” by H. C. Longuet-Higgins (*Nature*, **263**, 646; 1976) there are some errors in the figures. In Fig. 3 the top left-hand number should be -9 ; in Fig. 4 the second x in the left-hand column should be a z ; in Fig. 7 the trilled minim should be prefixed by a $\#$.

Errata

In the article “Male emigration and female transfer in wild mountain gorilla” by A. H. Harcourt et al. (*Nature*, **263**, 227; 1976) the second author should be K. J. Stewart and not as printed.

In the article “Axial, magnetron, cyclotron and spin-cyclotron-beat frequencies measured on single electron almost at rest in free space (geonium)” by R. Van Dyck, Jr, P. Ekstrom and H. Dehmelt (*Nature*, **262**, 776; 1976) $(n + \frac{1}{2})\nu_c$ in the first equation should read $(n + \frac{1}{2})(\nu_c - \nu_m)$. In the acknowledgment, for Schwingberg read Schwinberg.

Nature Index and Binders

The **Index** for 1975 is now available, price £2.25. Copies of the 1974 index are still on sale, price £3.00. **Binders** for the journal are also available at £8.00 for four (a year of *Nature* fits into four binders).

Postage is included in the above prices. Orders should be sent, accompanied by remittance to Macmillan Journals Ltd, Brunel Road, Basingstoke, Hampshire, England.

reviews

Secrets of the Earth's core

Thomas H. Jordan

The Earth's Core. (International Geophysics Series.) By J. A. Jacobs. Pp. viii+253. (Academic: New York and London, 1976.) £8.50; \$22.

THE Earth is stubborn in yielding her secrets, and locked most tightly in her depths are the mysteries of her core. The core is separated from us by nearly 3,000 km of rock, so it is no wonder that our papers about its composition, state and dynamics are often prefaced by words like 'assumption', 'hypothesis' and 'paradox'. Pressures within the core are as yet inaccessible by static laboratory apparatus, but fall short of the domain in which the elegant theories of degenerate electron gases may be applied. Nearly everyone agrees that iron and nickel are the dominant atomic species in the core, but further statements about its composition depend heavily on models for the Earth's early history, a subject perhaps still better suited for religious thinkers than natural philosophers. The important questions of core dynamics seem to provide the most secure remaining refuge for the back-of-the-envelope, order-of-magnitude geonomist. For example, the average heat flux across the core-mantle interface is one of the great unknown quantities of terrestrial physics, a number whose demonstrated bounds are virtually useless.

Therefore, few geoscientists would be surprised to learn that, until now, the Earth's core has not been the title subject of a scholarly treatise. This deficiency has been remedied by Professor Jacobs, who has produced a very readable monograph of 253 pages. His narrative sketches the lines of evidence which, when suitably tangled, form the web of our understanding about the core. His treatment of this subject has been divided into five chapters the titles of which outline the content of the book: general physical properties of the Earth; origin of the core; thermal regime of the Earth's core; Earth's magnetic field; constitution of the core. As a bonus he concludes with a sixth chapter on the cores of other planets, paying due homage to the current emphasis on comparative planetology. This last chapter also serves to cheer us up: at least we Earth scientists know

this planet has a core, a conclusion that is uncertain for even the best studied of our neighbours.

The book is comprehensive: space is given to nearly every subject of current interest, although few are treated in any depth. Particularly valuable are the author's discussions of recent attempts to model the physical parameters of the core using seismic methods, his summary of the controversy that has surrounded the so-called Kennedy-Higgins paradox, and his synthesis of the evidence regarding core formation. The chapter on the magnetic field is adequate, although a more thorough treatment of the dynamo problem could have been attempted.

Throughout the book the mathematical level is very elementary, long derivations are absent, and equations are generally used only in a narrative

fashion. In spite of this lack of fancy mathematics, or perhaps because of it, the subject matter is modern and the information content is high. It is a good reference for specialists engaged in core research who wish to know what other specialists are up to. The bibliographies which follow each chapter are excellent, with nearly seven hundred references (including multiple citations), over half of which have been published since 1970.

This book should be an excellent text for graduate courses dealing with the internal constitution of the Earth. It will be interesting to see if, in five or ten years, this is still true. □

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Introduction to physiological psychology

Brain and Behaviour: A Textbook of Physiological Psychology. By Hugh Brown. Pp. x+413. (Oxford University: New York and London, March 1976.) £9.25.

THIS introduction to physiological psychology has grown out of a series of lectures for undergraduates. The author initially grapples with the problem of providing a balanced grounding in the principles of nervous function and I think he succeeds in his aim of holding the attention of the reader while proceeding at a somewhat breathless gallop through the first half of the book. The text is chatty and is liberally punctuated with anecdotes, photographs and excellent illustrations. Jargon is kept to a minimum and there is a glossary of terms at the end, ranging from ablation and acalculia to Witzelsucht and the Young-Helmholtz theory. Each chapter ends with a few well chosen suggestions for further study.

The reader is then propelled rapidly through, and sometimes over, the second half of the book, which covers a selection of behavioural correlates of physiological functions. There are sections of about 20 pages each on motivation, emotion, consciousness, frontal lobe localisation, learning,

personality and psychopathology; and then on to glimpses of social and aggressive behaviour, wanted and unwanted actions of drugs; and the book comes to a close with a brief discussion of the mind-body problem. Comments on the composition of this section must, to some extent, reflect personal biases; I missed any discussion of recent attempts to link neurotransmitter systems with reinforcement mechanisms, and the neuropharmacology of mental disorders receives scant attention. There are occasional jarring lapses into simplistic clichés—for example, "if mental illness could be reduced to a chemical problem . . ."; and, incidentally, imipramine is not a monoamine oxidase inhibitor.

The book is largely successful in its aim: it is interesting but not indigestible. As an hors d'oeuvres the basic ingredients are well selected, nice to look at, and they whet the appetite. One big snag is the price—an hors d'oeuvres at £9.25 probably means that most students would have to do without the main course.

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Behaviour genetics

The Genetics of Behavior. By Lee Ehrman and Peter A. Parsons. Pp. viii+390. (Sinauer Associates: Sunderland, Massachusetts; Freeman: Reading, June 1976.) £11.50.

BEHAVIOUR genetics is not easily organised into textbook form, as its literature covers a very diverse range of topics in a patchy fashion. Ehrman and Parsons have chosen to group their material according to experimental approach and experimental animal. There are chapters on 'single genes and behaviour', 'many genes and behaviour', 'Drosophila', 'Rodents', 'other creatures', 'man—continuous traits', 'man—certain discontinuous traits', and so on. Their decision is reasonable but it presents them with problems, for such groupings are not the best for bringing out the main questions to which behaviour genetics should be applied. This book was written just too early to have taken account of the sociobiology upsurge. Here, for example, we have an infinity of attractive theories confidently proposing a genetic basis for a huge range of complex social behaviour. Yet we know next to nothing about the way the simplest behavioural potential is encoded in genetic terms. This main question quickly breaks down into a cluster of questions concerning genes and behavioural development, and gene environment interactions. Of course, these and other major questions do emerge from Ehrman and Parsons' book, but I think they should have been more strongly accented, to give some structure to their review of the very disparate literature, only a few of whose studies really lead somewhere.

This will, however, be a most useful textbook; it is certainly the best since Fuller and Thompson produced the first complete survey of behaviour genetics in 1960. Ehrman and Parsons write very well, their style is generally light and attractive. They begin with a chapter of essential genetics and I would judge that even students with no biological background could then go on with little trouble. The examples they cite are described thoroughly, with good illustrations and tables. Although I might quibble with the balance in places, they have certainly drawn together a scattered literature with great skill. Both authors are primarily geneticists and at times, I find the behavioural component lacking in depth. For example, their accounts of Dilger's work on hybrid parrots and Sharpe and Johnsgard's duck hybrids miss some of the points.

The chapters on human behaviour genetics are very good. The real test

here is the treatment of the genetics of intelligence issue and this is the best modern account I know, steering a very clear and reasonable course, missing none of the genetical or social issues.

Ehrman and Parsons end with a chapter on the evolutionary implications of behavioural changes, and then finally give their appraisal of future directions for behaviour genetics. I don't really share their conviction that it will emerge as a distinct discipline, but genetics will remain a vital technique in tackling some of the most fundamental behavioural problems. This textbook can be warmly recommended to teachers and students alike.

Aubrey Manning

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Ice on the land

Glaciers and Landscape: A Geomorphological Approach. By David E. Sugden and Brian S. John. Pp. viii+376. (Edward Arnold: London, April 1976.) Boards £12; paper £5.95.

GLACIOLOGY and geomorphology are typical of many scientific subjects the early disciples of which, with a minimum of equipment, made bold observations from patient work almost always pursued in isolation. Since 1950 the pattern has changed to team work, sophisticated equipment, the sharing of investigation areas and special publications. Devotees from all industrialised nations meet regularly to learn of progress and methods, and to swap yarns of experience, always told with humour and understatement by those who, particularly in these two disciplines, raise their eyes to the hills from 'whence cometh their health'. Laboratory work has provided specific laws but the wide range of variables in natural processes in the field always raises new problems in the application of these laws to the observed landscape.

Glaciology is advanced primarily by physicists whereas geomorphology is lead by physical geographers. The authors are reputable glacial geomorphologists who appeal for investigations to link these two aspects more closely. The book systematically sets out a brief for the geomorphologist to recognise the scope of the ice problem and summarises what the glaciologist has done to assist.

There are five parts, each of three chapters. The first part, 'Glaciers and Glacier Dynamics', gives the basic properties of ice, the environmental factors influencing it, the morphology of

glaciers both *en masse* and in surface structure. Fundamental quantitative relations are limited to this first part.

'Glacier Distribution in Space and Time' provides a succinct summary of the ice on the Earth's surface and its fluctuations. The authors rightly criticise the overdramatisation by the media of the relation between glaciers and climate and conclude that reliable assessment of this relationship can only be made for high frequency events of low magnitude since precise data is only available for minor oscillations over the past few decades. The random element in oscillatory behaviour becomes more marked as the time scale increases.

The next two parts, 'Glacier Erosion' and 'Deposition', each give a chapter on process followed by ones on landforms and landscapes. Rock abrasion, meltwater transport and deposition of rock detritus, the characteristics of tills and the complex end-products of both processes are economically described. Meltwater phenomena extend well beyond the margins of present ice but the authors sensibly limit their account to the present glacierised phase.

The final part, 'Meltwater, a glacial subsystem', discusses a component in all glaciers that in recent years has received much attention from glaciologists and engineers. It is a very creditable account of an aspect that most authors have combined with erosion and deposition. As in the first part of the book, there is already new material to be incorporated, a common difficulty for authors in a subject developing so rapidly.

Each chapter ends with suggested 'Further Reading' and there is an index at the end of the book, preceded by 25 pages of references, since the authors have patiently sifted an extensive range of literary and photographic material, very well drafted and printed in the book. Almost every page has a diagram, a photograph, a table, a schematic model; and although the models are entirely qualitative and the authors admit that some will rapidly be superseded, most are new and will be welcomed by students being increasingly taught classifying systems.

Few books are better presented for the price and none more captivating to the reader interested in the fascinating phenomenon of ice on the land.

H. Lister is Reader in Physical Geography at the University of Newcastle upon Tyne, a member of the International Glaciological Society and of the British Geomorphological Research Group, and escapes to mountains and glaciers whenever possible. **H. Lister**

Dr Lister is a Lecturer in the Department of Geography at the University of Newcastle-upon-Tyne, UK.

obituary

Eric Higgs, who died in Cambridge on September 23 after a long illness, will be remembered especially for his contribution to economic prehistory over the past decade. He became an archaeologist at a time of life when most think only of retirement, after a first career as an economist and a second as a hill farmer. The impact that he made in his third career, as a research and teaching archaeologist at Cambridge University, is all the more remarkable for the fact that his health was poor—intermittently at first, but increasingly so with time.

He found prehistoric archaeology dominated by the belief that the key to understanding early man rested in the study of his artefacts and of the cultural groupings based on artefactual styles: the relationships between the prehistoric cultures were the cornerstone of the subject, largely divorced from the natural environment, and the ordinary economic life of prehistoric communities had been relegated by most prehistorians to a comparatively minor role in the history of early man. The study of prehistoric subsistence consisted of what he termed a 'horrid porridge' of specialist reports which, although embodying a considerable degree of technical competence in the analysis of biological data, usually did little to build an integrated picture of man's relationship with his environment. To Higgs, the emphasis on the importance of cultural data was fundamentally misleading, in that it obscured basic economic motives, such as the need for food, which were and are the vital core of human behaviour. By assigning subsistence to a central place in prehistoric archaeology, he did much to establish economic prehistory as an independent discipline based on its own concepts and supported by its own laboratory and field techniques.

These interests crystallised during his fieldwork in Greece (Epirus) in the

middle and late sixties. In 1967 he was appointed director of a British Academy Major Research Project to study the origins and early history of agriculture. From then until his death he presided over a hectic decade of original and fundamental research and fieldwork, in areas as diverse as Alaska and Israel, on periods as widely separated as the Middle Palaeolithic and the Mediaeval.

This research was important in two ways: a theoretical contribution to the study of prehistoric subsistence in general and early agriculture in particular, and methodological developments in fieldwork. His project undertook a long overdue reassessment of the basic concept of animal and plant domestication. He argued that it was unrealistic to expect that human subsistence in prehistory would divide neatly into hunting and farming; instead, there would have been a series of relationships between prehistoric men and their plant and animal resources. Prehistorians have yet to appreciate fully the implications of this reassessment, for they have yet to accept that when and where agriculture originated cannot be confined to a convenient postglacial Garden of Eden: or that its origins had better be re-defined on more sensitive and complex criteria.

Of the new techniques he introduced, some, such as froth flotation for retrieving botanical remains from habitation sites, or site catchment analysis for quantifying economic potential of the areas exploited from prehistoric settlements, were culled from other disciplines, and modified; others, such as sieving techniques for recovering faunal samples, were already in occasional use but have since become an integral feature of modern palaeoeconomic investigations. This fieldwork, embodied in two major publications—*Papers in Economic Pre-history* and

Palaeoeconomy—was conducted and published within five years and was usually undertaken on field budgets which would have confined many another expedition to the armchair. Higgs even made his students pay 5 shillings a day for the privilege of working 8-h on-off shifts (under floodlights if necessary) at the site. (Hammond Innes did his fieldwork for the novel *Lefkas Man* by watching Higgs in action in Greece.)

Inevitably, his research aroused much debate and criticism. Many of his colleagues felt uneasy about his conviction that prehistoric archaeology had hitherto been concerned with only trivial aspects of past human behaviour and few shared his sublime indifference to the vagaries of ceramic and lithic styles. Yet the simplicity of Higgs's viewpoint was its strength, for it provided a direct challenge to an ageing conceptual framework which had denied the biological basis of prehistoric human behaviour. As Higgs said, "did Man Make Himself and control his own destiny as Childe had taught, or did he, like Topsy, just grow?"

Those who knew him personally, could not fail to recognise him as a man of remarkable character. He was an utter individualist, with scant regard for convention in either his professional or his personal life. It was in keeping with the man that he merged both, and shared them with his students to a remarkable degree; in return he expected and generally received a degree of commitment that often surprised even those who gave it. Eric Higgs was a remarkable man, in particular a gifted and riveting teacher. Prehistoric studies in general, as well as the personal and professional lives of those who knew him, are much the poorer for his death.

Graeme Barker
Robin Dennell

Benjamin Franklin Howell, Professor Emeritus of Geology and Palaeontology, at Princeton University, died on May 28 at the age of 85.

Howell was born on September 30, 1890, in Troy Hills, New Jersey. At the age of 18 he entered Princeton University where he received his Bachelor's degree in 1913, a Master's degree in 1915, and a Doctor of Philosophy degree in 1920, all in the Department

of Geology. He remained at Princeton where he was appointed as Assistant Professor in 1920 and became Professor of Geology and Palaeontology in 1947. He also served as Curator of Palaeontology and Stratigraphy at Princeton from 1924 until his retirement in 1959.

In addition to his work at Princeton, Howell was engaged in adult education as Professor of Geology at the Wagner

Free Institute of Science in Philadelphia, Pennsylvania, from 1928 to 1947. He also served as part-time Curator of Palaeontology at the Academy of Natural Sciences in Philadelphia from 1937 to 1947 and as a Visiting Lecturer in Palaeontology at the University of Pennsylvania.

He was a recognised authority on the palaeontology of the Cambrian Period, the earliest geological period of abun-

dant marine invertebrates. He was one of the founders of the International Palaeontological Union, and served as its Secretary in 1936-1937 and as its Vice-President in 1938. He was also an Honorary Member of the Geological Society of London as well as of the Palaeontological Society of India. At various times he had served as a consultant for the United States Geological Survey, the United States National Museum, the Geological Survey of Canada, the Canadian National

Museum, and the geological surveys of Vermont and Montana.

In America Howell was an active member and officer in both palaeontological and geological societies: as a Fellow of the Palaeontological Society he served as its Secretary from 1931 until 1939 and as President in 1944, and was a member of several of its committees concerned with Cambrian fossils and stratigraphy. As a Fellow of the Geological Society of America he was elected Vice-President in 1945.

During the early 1950s he was Chairman of the Cambrian section of the National Research Council's Committee on Stratigraphy.

During his long career Howell was one of the more prolific writers in the geological profession. His bibliography includes more than 200 titles of geological and palaeontological contributions, dealing mainly with Cambrian marine invertebrates, especially trilobites.

Erling Dorf

announcements

Appointments

Dr Barrie Vernon-Roberts as Professor of Pathology at the University of Adelaide.

Mr A. R. Haly as chief of CSIRO Division of Textile Physics.

Dr D. G. Murchison to a Chair of Organic Petrology at the University of Newcastle upon Tyne.

Professor J. Heslop-Harrison as a Royal Society Research Professor, to be taken up at the University of Wales, Aberystwyth.

Professor G. W. Kenner as a Royal Society Professor at the University of Liverpool.

Professor J. L. Gowans of the University of Oxford will take over as the new secretary of the MRC in April.

Meetings

November 17, **Cumulonimbus Convection**, London (T. M. R. Whitwell, James Glaisher House, Grenville Place, Bracknell, Berkshire RG12 1BX, UK).

January 3-5, **Mathematical Aspects of Homogeneous and Heterogeneous Combustion**, Cranfield Institute of Technology, Bedford (The Secretary, The Institute of Mathematics and its Applications, Maitland House, Warrior Square, Southend-on-Sea, Essex, UK).

January 6-7, **Respiratory Gas Transport**, London (The Secretary, The Institute of Mathematics and its Applications, Maitland House, Warrior Square, Southend-on-Sea, Essex, UK).

January 17-22, **Gondwana**, Calcutta (Organising Committee, IV International Gondwana Symposium, The Geological Survey of India, 27, Jawaharlal Nehru Road, Calcutta-700016, India).

January 31-February 2, **Cadmium**, San Francisco (The Cadmium Association, 34 Berkeley Square, London W1X 6AJ, UK).

February 7-8 (Chicago) and February 14-15 (Brussels), **Laboratory Testing**

Person to Person

Applications for the Victor Heiser Awards for Leprosy Research are invited to apply to Ms Caroline R. Stanwood, Director, Heiser Fellowship Program for Research in Leprosy, 1230 York Avenue, New York, New York 10021. The awards are for: Postdoctoral Research Fellowships, \$10,000-14,000, applications from individuals or heads of departments, Visiting Research Awards (for specific research at distant institutions), Research Grants (possibly available for specific programmes).

Wanted: 3- or 4-bedroom furnished house in central London, preferably Ealing, from August 1, 1977 to July 30, 1978, in exchange for house in Halifax. Replies to Carl Abbott, M.D., Department of Medicine, Camp Hill Hospital, Halifax, Nova Scotia, Canada B3H 3G2.

M.I.T. professor offers a 12-room fully furnished Victorian home in Brookline, Mass., in exchange for a 7-room (minimum) home within reasonable commuting distance of central London for one year beginning July 1, 1977. Brookline is very near Harvard, M.I.T., and major hospitals: has excellent schools and public transportation. Please reply to Dr Harvey Lodish, M.I.T. 16-435, Cambridge, Mass., 02139.

for Cancer (Robert S. First, Inc., 405 Lexington Avenue, New York, New York 10017).

March 23-25, **Nuclear Physics**, Guildford (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1X 8QX, UK).

April 4-7, **Atomic and Molecular Physics**, Reading (K. Codling, Conference Secretary, J. J. Thomson Physical Laboratory, Whiteknights, Reading RG6 2AF, UK).

April 4-7, **Low Temperature Biological Microscopy**, Cambridge (Dr P. Echlin, The Botany School, University of Cambridge CB2 3EA, UK).

May 8-12, **Psychoneuroendocrinology**, Atlanta (Dr Richard P. Michael, Department of Psychiatry, Emory University School of Medicine, Atlanta, Georgia 30322).

July 13-15, **Processing, Structure, Properties and Performance of Polymers**, Nottingham (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1X 8QX, UK).

July 24-30, **Optimal Development and Management of Groundwater**, Birmingham (Dr J. W. Lloyd, Organising Secretary, IAH Symposium, Department of Geological Sciences, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK).

August 22-24, **Scientific Instruments: Their Social and Economic Settings**, Greenwich (Organising Secretary, Department of Navigation and Astronomy, National Maritime Museum, Greenwich, London SE10 9NF, UK).

September 4-8, **Low Energy Ion Beams**, Salford (The Meetings Officer, The Institute of Physics, 47, Belgrave Square, London SW1X 8QX, UK).

September 19-24, **Behavioural Development**, Pavia, Italy (Secretary to the 4th Biennial Congress of the International Society for the Study of Behavioural Development, The Institute of Psychology of the Faculty of Letters and Philosophy of the University, Corso Strada Nuova, 65, 27100-Pavia, Italy).